Evaluation of new diagnostic technologies for rapid detection of urinary pathogens and their antibiotic resistances

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Abstract

Background: Most urinary tract infections (UTIs) are trivial; but complicated UTIs are a growing reason for hospitalisation in the UK, and are among the commonest sources of sepsis. Increasing resistance among uropathogens complicates treatment and drives wider empirical use of previously-reserved antibiotics. Rapid precise detection of pathogens and resistances, without culture, might better guide early therapy in deteriorating UTI patients.

Methods: Two approaches were evaluated: i) MALDI-TOF mass spectrometry for direct identification of pathogens from urine together with multiplex, tandem PCR (MT-PCR) for resistance gene profiling. MALDI-TOF was also explored for rapid detection of β-lactamase activity in bacteria harvested from urine; ii) MinION sequencing for bacterial and resistance gene identification, again directly from urine. As background, an epidemiological surveillance of uropathogens from the Norfolk and Norwich University Hospital in July and November 2014 was performed.

Results: Direct MALDI-TOF on urines could achieve rapid bacterial identification within 1.5 h and also allowed direct detection of extended-spectrum β-lactamase (ESBL) activity. MT-PCR showed satisfactory results in detecting the commonest resistance genes in Enterobacteriaceae directly from urines and cultivated isolates within 3 h. Weaker association was found between streptomycin resistance and *aadA1/A2/A3* genes. Fluoroquinolone-susceptible and -resistant *Escherichia coli* were distinguished by the melting temperatures of their *gyrA* product. MinION sequencing correctly identified uropathogens and their resistances in all urine samples within <5 h, without culture. Acquired resistance genes agreed with resistance phenotypes and closely matched Illumina sequencing, albeit with poor discrimination within some β-lactamase families (e.g. bla_{TEM}). Epidemiological surveillance showed *E. coli* predominant in all age groups and location types, with high resistance rates to amoxicillin and trimethoprim.

Conclusion: Either a MALDI-TOF plus PCR or a sequencing approach could significantly shorten the time required for microbiological investigation of urosepsis, allowing clinicians to adjust therapy before the second dose of a typical (i.e. q8h) antibiotic.

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Declaration

This thesis is the result of three years of research conducted at the Medical School Faculty of Health Sciences at the University of East Anglia in Norwich, UK. Throughout the period of the research, some of the contents concerning Nanopore sequencing have been published in the following article:

Schmidt K., Mwaigwisya S., Crossman L., Doumith M., Munroe D., Pires C., Khan A., Woodford N., Saunders N., Wain J., O'Grady J., Livermore D. Identification of bacterial pathogens and antimicrobial resistance directly from clinical urines by nanopore-based metagenomic sequencing. J Antimicrob Chemother (2017) 72 (1): 104-114.

Therefore, as the main author, I used some parts of the published article in my thesis. I declare that this thesis comprises only my original work towards the PhD and no portion of the work referred to in this thesis has been submitted in support of an application for another degree.

Research ethics approval for collected clinical samples used in this study was not required as these were excess samples from those submitted to the microbiology laboratory for routine analysis (no additional 'samples taken' were required). Treatment practice was not affected and clinicians did not receive results. Collected clinical samples were labelled with a study specific reference number, without patients' identifiable data. The study number was used to compare obtained results (bacterial identification and phenotypic profile) with those from the Norwich and Norfolk University Hospital routine testing.

The epidemiological surveillance of urinary pathogens from the NNUH in July and November 2014 performed in this study contained general information about epidemiology, and demographics to perform statistical analysis. No clinical information of the patients was collected.

Abbreviations

A&E	Accident and Emergency
AALS II	Anionic Acid Labile Surfactant II
ABS	Asymptomatic Bacteriuria
ACT	Acetonitrile
AMC	Co-amoxiclav (Augmentin)
AMX	Amoxicillin
AMU	Acute Medical Units
ARMA	Antimicrobial Resistance Mapping Application
ARG-Annot	Antibiotic Resistance Gene Annotation database
ASP	All Small Particles
AssU	Assessment Unit
AU	Admissions Units
BAG	Bag Urine
BC	Bacterial Count
BD	Twice daily
BLAST	Basic Local Alignment Search tool
BTS	Bacterial Testing Standard
BSAC	British Society for Antimicrobial Chemotherapy
CARD	Comprehensive Antibiotic Resistance Database
CA UTI	Community Acquired Urinary Tract Infection
CAT	Catheterized Urine
CAZ	Ceftazidime
CFU	Colony Forming Units
CIP	Ciprofloxacin
CLX	Cephalexin
CLED	Cysteine Lactose Electrolyte Deficient
CLSI	Clinical and Laboratory Standards Institute
GEN	Gentamicin
CPD	Cefpodoxime

CRO	Ceftriaxone
CSU	Catheterised Stream Urine
CXM	Cefuroxime
CU	Clinical Urine
cUTI	Complicated Urinary Tract Infection
ESBL	Extended-spectrum β-lactamase
ETP	Ertapenem
EUCAST	European Committee on Antimicrobial Susceptibility Testing
NIT	Nitrofurantoin
FA	Formic acid
FEP	Cefepime
FOS	Fosfomycin
FOX	Cefoxitin
GENI	Genital
GP	General Practice
HCCA	α-cyano-4-hydroxy cinnamic acid
H_IN	Hospital Inpatients
H_OUT	Hospital Outpatients
ITU	Intensive Therapy Unit
LB	Luria-Bertani
MALDI-TOF MS	Matrix-Assisted Laser-Desorption/Ionization Time of Flight Mass Spectrometry
MEM	Meropenem
MIC	Minimal Inhibitory Concentration
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MSSA	Methicillin-Susceptible *Staphylococcus aureus*
MSU	Mid-Stream Urine
NNUH	Norfolk and Norwich University Hospital
O/N	Overnight
ONT	Oxford Nanopore Technology
OH	Other Hospitals
PBS	Phosphate-buffered saline

RBC	Red Blood Cell
RQ	Resistance Quotient
SCU	Spiked Clinical Urine
SDS	Sodium Dodecyl Sulphate
SPUB	Supra Pubic Aspirate
SRST2	Short Reads Sequencing typing software
STET	Immediately
SU	Spiked Urine
TDS	Three times per day
TFA	Trifluoroacetic acid
TZP	Piperacillin
URIN	Urine
UTI	Urinary Tract Infection
VRE	Vancomycin-Resistant Enterococcus
TMP	Trimethoprim
WBC	White Blood Cell
WIMP	‘What’s in my pot’ software
WGS	Whole Genome Sequencing
QDS	Four times a day

Chapter 1

INTRODUCTION

1.1 Anatomy of the urinary tract

The urinary tract is made up of the kidneys, ureters, bladder and urethra (Figure 1). The kidneys belong to the upper urinary tract along with the ureters, and are located in the abdomen, on either side of the spine. The ureters are thin tubules that carry urine from kidney to the bladder. The remaining structures comprise the lower urinary tract. The bladder a balloon-shaped organ, is located in the pelvis of women while in men it lies above the prostate gland. The urethra is the tube through which urine exits the bladder.

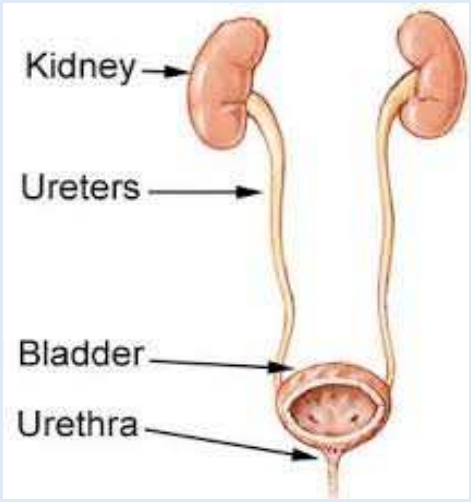

Figure 1. Principal anatomical components of the urinary tract.

1.2 Urinary tract infections (UTIs)

An inflammation of the urinary tract can occur in urethra (urethritis), bladder (cystitis), kidneys and renal pelvis (pyelonephritis), epididymis (epididymitis) or prostate gland (prostatitis). In extreme cases infection overspills to the bloodstream and manifests as sepsis, severe sepsis or septic shock. For clinical management, UTIs are classified into four categories: asymptomatic bacteriuria, uncomplicated UTI, complicated UTI and catheter-related UTI. Depending on the location of the infection, symptoms vary ranging from mild irritation during voiding to sepsis (Table 1). The bacterial counts for clinically relevant diagnostic of UTI are presented in Table 2.

Table 1. Clinical symptoms and signs of upper and lower urinary tract infection.

Lower urinary tract infections	Upper urinary tract infections
Dysuria	Systemically unwell
Burning with urination	Nausea and vomiting
Frequency of urination	Uncontrollable shivering
Malodorous urine	Hypotension or shock
Suprapubic, and rectal (men) pain	Loin pain and tenderness in the upper back and sides
Normal temperature	
Haematuria	Fever
	+/- Features of lower urinary tract infection

(Field, 2010)

Table 2. Laboratory correlates of different form of urinary tract infection.

Categories	Clinical diagnosis	Laboratory correlated
1	Asymptomatic bacteriuria (ASB)	$\geq 10^5$ cfu/mL for women (in two consecutive voided specimen with the same species) and men (in one voided specimen with one species); $\geq 10^2$ cfu/mL for patients with indwelling catheter
2	Acute uncomplicated cystitis Acute uncomplicated pyelonephritis	>10 WBC/mm^3 $\geq 10^3$ cfu/mL >10 WBC/mm^3 $\geq 10^4$ cfu/mL
3	Complicated UTI (cUTI)	>10 WBC/mm^3 $\geq 10^5$ cfu/mL for women $\geq 10^4$ cfu/mL for men or in straight catheter urine for women
4	Catheter-related UTI	>10 WBC/mm^3 $\geq 10^3$ cfu/mL

(Naber *et al.*, 2006; Nicolle, 2006; Ipe *et al.*, 2013; Grabe, 2015)

1.2.1 Asymptomatic bacteriuria (ASB)

Asymptomatic bacteriuria is defined as the presence of significant numbers (>10^5 cfu/mL for women and men or >10^2 cfu/mL for patients with indwelling urethral catheter) (see Table 2) of a single organism in urine in the absence of symptoms indicating infection (see Table 1). The prevalence of ASB among the general population is estimated at 3.5% (Foxman, 2002) and increases with the age. Among elderly (>65 years old) living independently the ASB rate is three times greater in women (16% to 18%) than men (6%) (Nicolle *et al.*, 2005). Among elderly people women living in nursing homes, ASB rates range from 17% to 55% and among elderly

men from 15% to 31% (Nicolle *et al.*, 2005). ASB is even more prevalent among residents of long-term facilities, at up to 75% of institutionalized women and 52% of men (Ipe *et al.*, 2013). Other groups with substantial ASB include pregnant women (1.9-15%) (Ipe *et al.*, 2013), also transplant and diabetic patients, whose have 2-fold to 4-fold higher incidence rate of ASB compared with non-diabetic patients (Foxman, 2002).

1.2.2 Uncomplicated UTIs

Uncomplicated UTIs occur in lower and upper urinary tracts in healthy women who have no underlying structural or neurological lesions of the urinary tract, no history of recent instrumentation and no other systemic diseases predisposing them to bacterial infection. Infection is usually caused by single pathogens, principally *Eschericha coli*. Uncomplicated UTIs are encountered most frequently in young, sexually-active women (Mehnert-Kay, 2005), and manifest usually as acute uncomplicated cystitis with colony counts $\geq 10^3$ cfu/mL. Sometimes infection spreads from bladder to the upper urinary organs (kidneys or renal pelvis) and manifests as uncomplicated pyelonephritis with a diagnostic threshold $\geq 10^4$ cfu/mL (see Table 2) along with clinical symptoms. Symptoms of upper UTI usually involve fever and pain in the upper back (see Table 1) even without the symptoms typical for lower urinary infections.

Early studies by Kass (Kass, 1960) demonstrated that asymptomatic bacteriuria during pregnancy is associated with an increasing risk of developing pyelonephritis due to mechanical compression of the enlarging uterus (Schnarr & Smaill, 2008). The incidence of pyelonephritis during pregnancy ranges from 0.5 to 9% (Bookallil *et al.*, 2005; Sharma & Thapa, 2007; Hill *et al.*, 2005) and it is associated with many complications including bacteraemia, renal disease, hypertension, preterm labour and low birth weight (Hill *et al.*, 2005; Schnarr & Smaill, 2008).

1.2.3 Complicated UTIs (cUTIs)

Complicated UTIs occur in patients in whom there are residual inflammatory changes following recurrent infection and instrumentation or with anatomical, structural or functional abnormality due to intrinsic or extrinsic factors (Mazzulli, 2012). Complicated UTIs may occur with or without clinical symptoms common for lower and upper urinary tract infections (see Table 1). Infection is usually caused by *E. coli*, but other pathogens including non-fermenters (*Pseudomonas aeruginosa*) and Gram-positive cocci (e.g. staphylococci or enterococci) may be important. *E. coli* isolated from patients with complicated urinary infection have a lower prevalence of virulence genes and less phenotypic expression of virulence factors compared with *E. coli* isolated from uncomplicated infections (Johnson *et al.*, 1988; Johnson *et al.*, 1987; Benton *et al.*, 1992; Sandberg *et al.*, 1988; Nicolle, 2001).

Complicated UTIs can arise in both sexes regardless of age; however UTIs in men are usually considered complicated (Naber *et al.*, 2001; Naber *et al.*, 2006) and are frequently associated with acute or chronic pyelonephritis, prostatitis or perinephric and renal abscesses.

UTIs are a growing cause of hospitalization in the UK, with 67 emergency admissions per 100,000 population per quarter on average, mostly of elderly patients (see Figure 2) (NHS, 2014). In recent years the number of *E. coli* bloodstream infections has significantly increased with 35,676 incidences recorded in England in 2014-15 (Figure 3) over 60% with an urinary origin (PHE, 2016b).

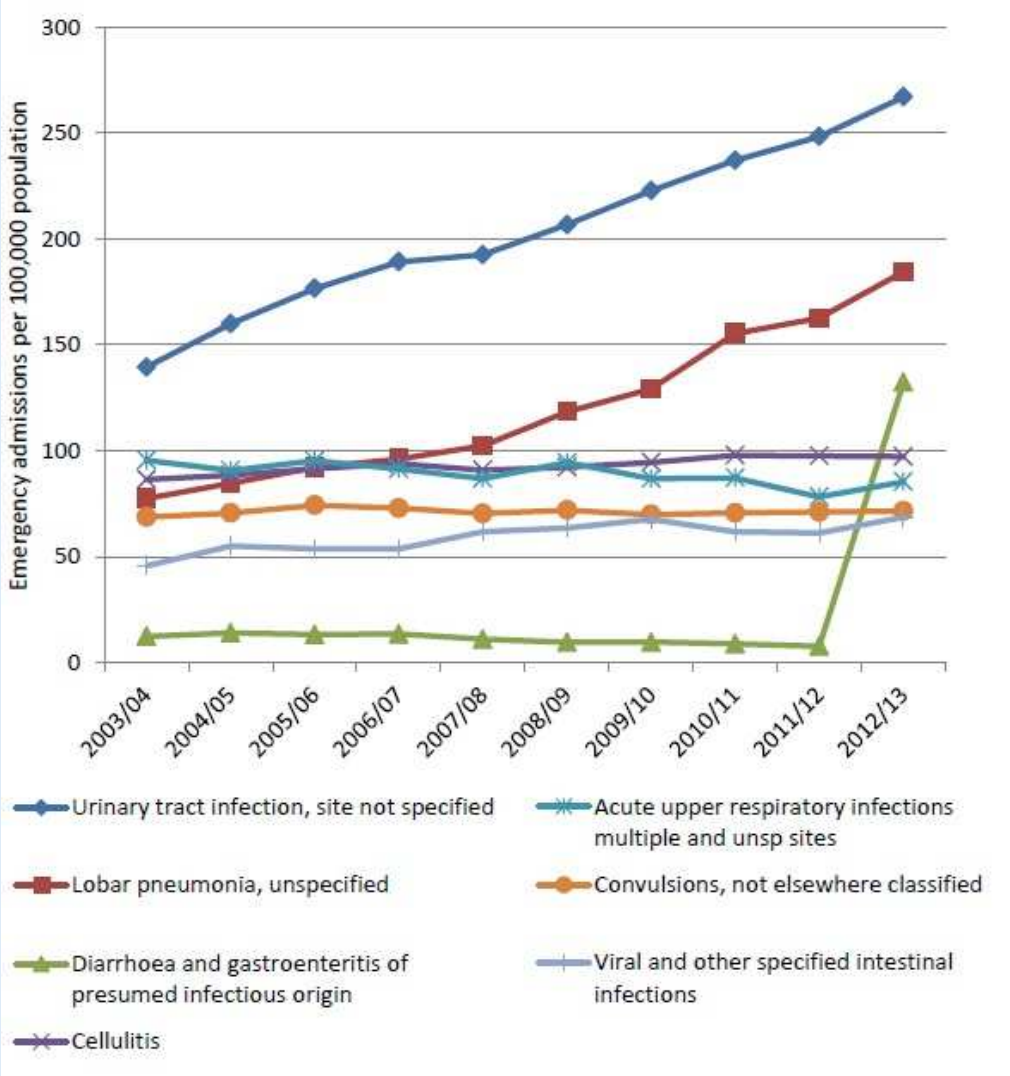

Figure 2. Growing rate of admission for UTI compared with other causes for hospitalization over time (NHS, 2014).

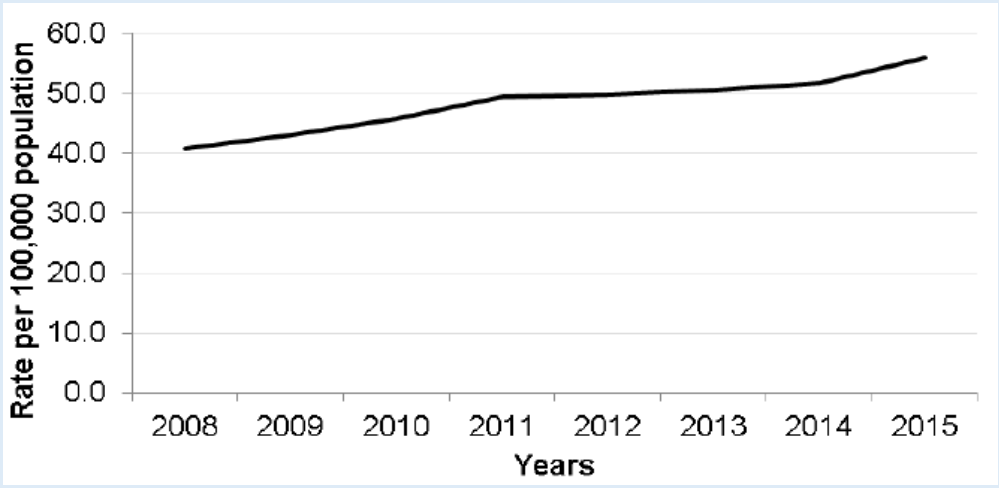

Figure 3. *E. coli* bacteraemia rate per 100,000 population in England 2008-2015 (PHE, 2016b).

1.2.4 Catheter-related UTIs (CA UTIs)

Catheter-associated urinary tract infections (CA) arise in patients with indwelling urinary catheters. A colony count $>10^3$ cfu/mL of one bacterial species in a single catheter urine specimen is microbiologically significant among patients with a urethral, suprapubic or condom catheter, even where this has been removed within the previous 48 h (Hooton *et al.*, 2010). From 15% to 25% of patients admitted to hospital undergo urinary catheterization (Hooton *et al.*, 2010; Warren, 2001) and a similar proportion of patients cared in nursing homes have long-term indwelling catheters (Godfrey & Evans, 2000). CA-bacteriuria can occur in patients with or without clinical symptoms and signs referable to urinary infections with the incidence rate of bacteriuria between 3%-10% per cathetered day (Rosser *et al.*, 1999; Warren *et al.*, 1978; Lo *et al.*, 2014).

Most catheter-associated UTIs derived from patient's own colonic flora (Tenke *et al.*, 2008) but long-term catheterization increases the risk of UTI caused by nosocomial pathogens (e.g. *Pseudomonas* spp.). The major risk factor of developing catheter-related bacteriuria is the duration of catheterisation. Short-term episodes (less than 7 days) mostly are asymptomatic and often caused by a single organism, while a long-term catheterization (more than 30 days) increases the risk of polymicrobial infection (Warren, 2001; Tenke *et al.*, 2008; Rosser *et al.*, 1999). Other risk factors associated

with CA-bacteriuria include lack of systemic antimicrobial therapy, gender (female), microbial colonization of the drainage bag, catheter insertion outside the operating room, catheter care violations, comorbidity or fatal underlying illness, older age, diabetes (Maki & Tambyah, 2001; Saint & Chenoweth, 2003; Rosser *et al.*, 1999).

Infection is usually caused by *E. coli*, but a wide variety of others pathogens might be isolated including *P. aeruginosa*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Stahylococcus epidermidis*, *Enterococcus* spp. and *Candida* spp. (Warren, 2001; Sedor & Mulholland, 1999). Bacterial adhesins initiate attachment by recognizing receptors located on the surfaces of the host cells or catheter. Once attached to the catheter internal or external surface, bacteria grow by initially forming microcolonies with a mature biofilm developing later (Jacobsen *et al.*, 2008). Bacteria within the biofilm produce exopolysaccharides which protects them from mechanical flushing by urine flow and other host defences whilst cell dormany protects from antibiotics (Tenke *et al.*, 2008). Treatment of asymptomatic CA UTIs is not recommended. Antibiotic treatment is administrated only for symptomatic infection.

1.2.5 Urosepsis

Urosepsis is defined as sepsis (septicaemia syndrome) arising from prior infections localized in the upper urogenital tract or male genital organs (prostate) (Wagenlehner *et al.*, 2007a) typically with bloodstream overspill. It may be associated with multi-organ dysfunction, hypo-perfusion or hypotension. Sepsis can develop from community- or nosocomial-acquired complicated UTI. However, in a study performed during the 1990s by Richards *et al.* it was noted that 23% of all cases of hospital-acquired sepsis were due to UTI and were mostly seen in catheterized patients (Richards *et al.*, 2000). Severe sepsis is most often associated with pulmonary and abdominal infections, but prior urinary tract infections account for about 5% cases (Hotchkiss & Karl, 2003). The prevalence of nosocomial urosepsis in urological patients in urology is high and in one study it was estimated at 12% (Bjerklund Johansen *et al.*, 2007). In general, urosepsis accounts for approximately 25% of all sepsis (Wagenlehner *et al.*, 2013). The mortality rate of urosepsis is high and ranges from 20-42% in high-risk group patients (Levy *et al.*, 2012; Rosser *et al.*,

1999; Tal *et al.*, 2005). These high-risk cases include patients with comorbid illnesses, advanced age, diabetes, immunosuppression (transplant), tumour receiving chemotherapy or corticosteroids and patients with acquired immunodeficiency syndrome. The commonest risk factor associated with developing urosepsis are structural or functional genito-urinary abnormalities. These include congenital or acquired obstruction (e.g. calculi, ureteric or urethral strictures or tumours), instrumentation (e.g. indwelling urethral catheters, nephrostomy tubes, urological procedures), impaired voiding (e.g. neurogenic bladder, cystocele), metabolic abnormalities (e.g. diabetes) and immunodeficiency (Kalra & Raizada, 2009).

Gram-negative bacilli account for majority of the cases of urosepsis. They include *E. coli* (50%), *Proteus* spp. (15%), *Enterobacter* spp. and *Klebsiella* spp. (15%), and *P. aeruginosa* (5%), while Gram-positive organisms are involved less frequently (15%) (Wagenlehner *et al.*, 2007b).

Effective antimicrobial therapy within the first hour of documented hypotension was associated with a survival rate of 80% in sepsis generally (Kumar *et al.*, 2006). Each hour of delay in the subsequent six hours was associated with an average decrease in survival by 8%.

1.3 Microbiological definition of UTI

A "significant" bacteriuria is conventionally defined as $\geq 10^8$ cfu/L or $\geq 10^5$ cfu/mL of one bacterial species in a clean-catch mid-stream urine as first proposed by (Kass, 1956)**.** Kass found that lower numbers of bacteria generally indicated contamination during sample collection, although bacterial loads of 10^4-10^5 cfu/mL were difficult to interpret. Nevertheless, approximately one-fourth of patients presenting with symptoms suggesting acute uncomplicated UTIs yield no bacterial growth on urine culture or counts between 10^2 - 10^4 cfu/mL (Gallagher *et al.*, 1965). Acute pyelonephritis can occur with lower bacterial counts, especially among some groups of patients (MacDonald *et al.*, 1957) e.g. children, pregnant women, and elderly populations with comorbid illnesses. McIsaac *et al*. (2005) reported that single culture-based methods miss more than one-half of asymptomatic bacteriurias among

pregnant women before 20 weeks gestation, and recommended culture in each trimester to identify more cases (McIsaac *et al.*, 2005).

Therefore, the microbiological criteria for the diagnosis of significant UTI have gradually been refined and depends on the patient group and types of bacteria detected (see Table 2).

1.4 Aetiology of urinary tract infection

UTIs are among the most frequent bacterial infections. It is estimated that there are about 150 million cases worldwide per annum (Stamm & Norrby, 2001); they account for over 8 million physician visits p.a in the USA (Schappert, 2008) and for 1-6% (Nazareth & King, 1993) of all consultations in general practice in the UK each year. The prevalence of UTIs depends on demographics, medical interventions and comorbidities (see Table 3). For anatomical reasons, women are more prone to develop UTIs than men. Most infections in adult men are complicated and related to abnormalities of the urinary tract. Approximately 50% of women experience uncomplicated UTI requiring antimicrobial therapy by the age of 30 (Foxman, 2002; Ikaheimo *et al.*, 1996). The recurrence rate is as high as 20-30% (Hooton, 2001) within 6 months of completing antimicrobial treatment. The incidence of UTI increases with the age for both genders. It is estimated that the incidence of UTIs per year in children ranges between 1-3% (Foxman, 2002); in premenopausal women it is 12% (Hagglund *et al.*, 1999); in elderly non-institutionalized women and men, 6-30%, and 11-13%, respectively (Wolfhagen *et al.*, 1990) while, in institutionalised elderly women, UTI rates range from 25-50% (Raz, 2011; Wolfhagen *et al.*, 1990).

UTIs among the elderly population (>65 years old) are very common, occurring both in community and long-term care settings. The clinical presentation of UTI range from asymptomatic bacteriuria to complicated UTI. Although most infections are asymptomatic or mild, severe infections can also be devastating, resulting in sepsis or death. Screening for a treatment of asymptomatic bacteriuria in elderly institutionalized residents of long term care facilities is not recommended (Ronald *et al.*, 2001) and treatment of asymptomatic bacteriuria does not reduce mortality or

prevent symptomatic episodes but increases side effect and antibiotic resistance (HPA, 2011.). Antibiotic therapy is only administrated for asymptomatic bacteriuria in the presence of factors potentially leading to complicated UTI and before urologic procedures during which mucosal bleeding is anticipated (Nicolle *et al.*, 2005). There are many factors that predispose older patients to develop symptomatic and complicated UTI (see Table 3). Chronic disease (e.g. Alzheimers and Parkinsons) and neurological conditions associated with cerebrovascular disease lead to impaired bladder empting (Nicolle, 2002). Urological conditions causing obstruction (e.g. stones, tumours) also increase the risk of developing UTI. The presence of urinary catheters and external urine collection devices increases the frequency of bacteriuria. Patients with comorbid diabetes with contributing factors including neurogenic bladder and poor glycemic control are at high risk of developing asymptomatic bacteriuria (Zhanel *et al.*, 1995). In postmenopausal women oestrogen deficiency corresponds to recurrent UTI (Stamm & Raz, 1999). In men, chronic prostatic disorders cause urinary symptoms and urinary retention.

Table 3. Major risk factors prone to developing UTIs.

Young population	**Elderly population**
Sexual activity Diaphragm and spermicidal use Use of antimicrobial agents Pregnancy	Chronic diseases and specific medications Anatomical or functional abnormalities of urinary tract Comorbid diabetes Institutionalization Presence of a urinary catheter Oestrogen level (women) Chronic prostate disorder (men) Poor health Vaginal disorders Urge incontinence
Delayed, incomplete postcoital voiding Recent UTI	

1.5 Epidemiology

More than 95% of uncomplicated UTIs are caused by a single bacterial pathogen (Lentz, 2009). The great majority- 75-90%- are caused by *E. coli* (Koksal *et al.*, 2017; Malmartel & Ghasarossian, 2016; Karlowsky *et al.*, 2011; Gupta *et al.*, 2001; Nicolle, 2013; Kahlmeter, 2003), however UTIs are caused by only a few *E. coli* types. Historically these were defined by serotyping and important type was e.g. O15:K52:H1 (Dalmau *et al.*, 1996; Olesen *et al.*, 1995; Johnson *et al*., 1999; Prats *et al.*, 2000; Phillips *et al.*, 1988). Sequence typing has now replaced serotyping, but still a few types dominate e.g. 025b-ST131 (Rogers *et al.*, 2011; Cagnacci *et al.*, 2008; Day *et al.*, 2016), ST69 (Matsumura *et al.*, 2012), ST73 (Alhashash *et al.*, 2016), ST95 (Ciesielczuk *et al.*, 2016). This might reflect their prevalence in the faecal flora, or reflect differences in virulence factors. Uropathogenic *E. coli* possess adhesive organelles (fimbriae or pilli) facilitating bacterial attachment on the luminal surface of the bladder epithelium and penetrating into epithelial cells (Ronald, 2002). A variety of virulence factors are associated with *E. coli* UTI. Johnson *et al.* (2005) showed that *E. coli* isolates from patients with pyelonephritis and prostatitis exhibited more virulence factors than those with cystitis (Johnson *et al.*, 2005). Certain virulence factors such as haemolysin (*hly* gene), necrotizing factor type 1 (*cnf1* gene) or class III P-pilli (*papGIII* gene) specifically favour the development of prostatitis (Ruiz *et al.*, 2002; Johnson *et al.*, 2005) whereas others such as type 1 fimbriae (*fim* gene) and *ireA*, the K2 *kpsM* variant, *cvaC,* and *ibeA* favour pyelonephritis (Johnson *et al.*, 2005).

Most non *E. coli* cases of uncomplicated UTI are caused by other Gram-negative bacteria such as *Proteus mirabilis*, *Klebsiella* and *Enterobacter* species (5-10%) (Gupta *et al.*, 2001; Karlowsky *et al.*, 2011; Kahlmeter, 2000; Stefaniuk *et al.*, 2016) and by Gram-positive bacteria such as *Staphylococcus saprophyticus*, *Enterococcus faecalis*, and *Streptococcus agalactiae* (group B strep) (5-15%) (Farrell *et al.*, 2003; Kim *et al.*, 2008)

Complicated UTIs from patients with indwelling catheters are more often caused by two or more pathogens, which are likely to be multi-drug resistant. Enterobacteriaceae (*E. coli*, *Klebsiella* spp., *Enterobacter* spp., and *Proteus* spp.) are

the predominant pathogens (15%), with *E. coli* remaining the commonest (25-50%) (Mazzulli, 2012; Lentz, 2009; Ipe *et al.*, 2013). Other frequent isolates include the non-fermenters *Pseudomonas aeruginosa*, which accounts for approximately 5% of infected patients with indwelling devices (Nicolle & Committee*, 2005; Nicolle, 2012). Gram-positive cocci are responsible for around 15% infections mostly involving *Enterococcus* species (Nicolle and Committee*, 2005; Nicolle, 2012).

1.6 Treatment

In general, antimicrobial treatment in UTI should be reserved only for patients with clinical symptoms and signs, except for asymptomatic bacteriuria (>10^5 cfu/mL) in pregnancy and in transurethral resection of prostate where mucosal bleeding is likely to occur (Nicolle & Committee*, 2005). Local epidemiology, resistance profiles, and patient factors (age, gender, medical interventions, and comorbidities such as diabetes, immunosuppression, catheterisation, neuromuscular disorders, renal transplantation) should be taken into consideration before deciding on antimicrobial therapy.

Clinical laboratories should be also aware of natural (inherent) resistance phenotypes profile of common uropathogens. For example *Proteus mirabilis* is naturally resistant to nitrofurantoin and colistin, *Enterobacter* spp., *Citrobacter freundii* are naturally resistant to ampicillin, amoxicillin, co-amoxiclav, first-generation cephalosporins and cefoxitin (Livermore *et al.*, 2001).

Monitoring antimicrobial resistances, combined with reasonable antibiotic therapy should help to reduce the rate at which resistance emerges and spreads. It should also seek to minimise the super-infection rate with *Clostridium difficile*. Guidelines for empirical treatment of UTIs need to be regularly reviewed and updated.

The first-line treatment in the UK for community-acquired uncomplicated cystitis is nitrofurantoin (50 mg 4 times per day (qds) or 100 mg modified-release twice daily (bd)) given as a three- or five-days course for women and seven-days for men. Trimethoprim (200 mg bd) remains an alternative in an areas where the resistance rate for *E. coli* is <20%. A third possible agent is pivmecillinam (400 mg immediately

(stet) then 200 mg tds for 3 days) or, if the patient has penicillin allergy cephalexin (500 mg bd) (ASC, 2016; APC, 2017; PHE, 2016c; CCG, 2016). Fosfomycin (3 g stet) might be an alternative if the above agents cannot be used (ASC, 2016).

If infection is associated with diabetes, an indwelling catheter, renal impairment, an abnormal urinary tract, immunosuppression or recent UTI it is recommended to extend the therapy to 7 days still using above antibiotics (ASC, 2016). If symptoms persist, urine should be send for microscopy, culture and susceptibility testing and a further three-day course of nitrofurantoin or trimethoprim is prescribed. Simultaneously treatment is adjusted later according to the susceptibility testing results.

Hospital-acquired acute pyelonephritis without penicillin allergy in the UK often is treated by a course of piperacillin/tazobactam (4.5 g three times per day (tds)) with step down to oral cephalexin (500 mg bd) or co-amoxiclav if the bacteria are sensitive. Alternatives are ciprofloxacin (500 mg bd) or co-amoxiclav (500/125 mg tds). In cases of severe complicated pyelonephritis with penicillin allergy gentamicin or ciprofloxacin (500 mg bd) are recommended (ASC, 2016; APC, 2017; PHE, 2016c; CCG, 2016). The total duration of treatment, including the oral step-down, is around 10-14 days.

Severe urosepsis without penicillin allergy is treated by a ten-day course of single dose of gentamicin plus piperacillin/tazobactam (4.5 g) or if the patient has penicillin allergy, gentamicin plus meropenem (1 g tds), which is likely to induce a less severe reaction than penicillin or ciprofloxacin (500 mg bd) are used for 14-21 days (ASC, 2016). More generally carbapenems (ertapenem, imipenem, meropenem) should be used only in extreme cases when pathogen is resistant to standard antibiotics.

Suspected acute prostatitis is treated by ciprofloxacin (500 mg orally twice daily) or trimethoprim (200 mg bd) for 28 days (PHE, 2016c), though both these are seriously threatened by resistance.

Lower UTI in pregnancy is treated by a seven-day course of nitrofurantoin (50 mg qds) during the first and second trimesters; cephalexin (500 mg bd) or trimethoprim (200 mg bd) are alternatives during the third trimester. In the case of severe sepsis ceftriaxone is recommended (PHE, 2016c). Before implementing antibiotic therapy, urine from the pregnant woman should be sent for routine examination and

susceptibility testing; antimicrobial choice should be reviewed when culture results become available.

1.7 Antibiotic resistance

Antimicrobial resistance is causing a serious global public health care threat. The rapid spread of bacterial resistance while few new antibiotics are being developed has led to pressure to develop stewardship and treatment guidelines so as to better manage our dwindling antibiotic resources.

Until recently, it was considered that the hospitals, and especially intensive care units, were the major source of bacterial resistance, however the spread of antimicrobial resistances in community settings is increasingly seen. Examples include the increase of ESBL-producing *E. coli* (Doi *et al.*, 2013; Fan *et al.*, 2014; Woodford *et al.*, 2004).

The rise of resistance in the community links to growing antibiotic consumption. In the four years 2010-2014 total antibiotic consumption in England rose by 6.5% (PHE, 2016a). The majority of antibiotic prescribing is in primary care though secondary care prescribes more broad-spectrum agents, which are more likely to drive antibiotic resistance than narrow-spectrum antibiotics. Changes in the community e.g. increasing day-care for children, institutionalization of care for the elderly population, antibiotic abuse and agricultural use of antibiotics create the ground for the spread antimicrobial resistance.

Although it is easy to assess resistance rates to antibiotics in nosocomial urinary infection, surveillance of community-acquired infections is a challenge. MacGowan *et al.* (1998) suggested that route testing of *E. coli* isolated from urine specimens over-estimates the rate of resistance among *E. coli* in the community (MacGowan *et al.*, 1998). This is because the majority of samples submitted to the laboratory for routine examination lack details on why the sample was sent and may be from treatment failures-which may fail owing to resistance. Moreover, some patients group e.g. the elderly are more likely to have specimens taken than others (Livermore *et al.*, 1998). To measure the true rate of resistance in the community it would be

necessary the use a clinical denominator (number of infected patients) not number of laboratory isolates (Livermore *et al.*, 1998), and to test the isolates from all these patients by a standardized laboratory method for both bacterial identification and susceptibility testing.

The prevalence of antimicrobial resistance in urinary pathogen varies widely depending on the patient severity of illness, demography (e.g. urosepsis *vs*. cystitis and elderly *vs.* young), geographic location, primary care or hospital patient care. Local and stratified antimicrobial susceptibility and resistance rates therefore should ideally be considered in making treatment decision, but are rarely available.

Antimicrobial agents are often categorized according to their principal mechanism of action. Those include (i) interference with cell wall synthesis (e.g. β-lactams and glycopeptides), (ii) inhibition of protein synthesis (e.g. macrolides, aminoglycosides and tetracyclines), (iii) interference with nucleic acid synthesis (e.g. fluoroquinolones and rifampicin), (iv) inhibition of a metabolic pathway (e.g. trimethoprim-sulfamethoxazole), and (v) disruption of bacterial membrane structure or function (e.g. polymyxins and daptomycin) (Tenover, 2006). Bacteria may manifest intrinsic resistance to antibiotic or may acquire resistance by mutations or gene transfer from other bacteria. Specific mechanisms include (i) acquisition of β-lactamase genes (e.g. extended-spectrum β-lactamase or plasmid-mediated *ampC* genes) that hydrolyse antibiotic agents, (ii) expression of efflux pumps that extrude antibiotics from the cell before they reach the target site, (iii) acquisition of genes for a metabolic pathway by passes the binding site of the antimicrobial agents, (iv) mutations or acquisition of genes that modify an antibiotic target site and (v) mutations that down regulate porin genes, thereby limiting access of antibiotics to the intracellular target sites. Bacterial exchange of genetic information, increasing the spread of resistance genes occurs through transformation, conjugation and transduction and facilitates accumulation of multidrug resistance.

The years 1960-1980 saw dramatic rises in the antibiotic resistance for *E. coli* to amoxicillin/ampicillin (Kresken & Wiedemann, 1986; Atkinson & Lorian, 1984). Over succeeding decades *E. coli* progressively acquired resistance to trimethoprim, quinolones, amoxicillin-clavulanate, cephalosporins and to a lesser degree

piperacillin/tazobactam i.e the drugs that are commonly used to treat severe and complicated UTIs in hospitals (Potz *et al.*, 2006).

Resistance to β-lactams is typically mediated by the acquisition of plasmid-encoded β-lactamases including classical penicillinases (e.g. TEM-1, TEM-2, or SHV-1); AmpC cephalosporinase enzymes (e.g. CMY-1, 2, 3, FOX-1, MIR-1, LAT-1, MOX-1), which escaped from the chromosomes of *Enterobacter* and *Citrobacter* spp. (Bush *et al.*, 1995) and ESBLs. ESBLs, which mediates hydrolysis of cephalosporins include mutations of TEM and SHV penicillinases and CTX-M enzymes, which escaped from *Kluyvera* spp. (Tenover, 2006).

Resistance to trimethoprim is largely mediated by acquisition of resistant DHFR enzymes, which functionally by pass the drug's target site (Eliopoulos & Huovinen, 2001). Other rare mechanisms to trimethoprim include (i) changes to the permeability barrier and/or efflux pumps, (ii) regulatoryn changes in these DHFR target enzymes (DHFR) and (iii) mutational or recombination changes in the target enzymes (Eliopoulos & Huovinen, 2001). Presently, resistance rates among *E. coli* in the UK to trimethoprim and amoxicillin/ampicillin are around 30-40% and >50% isolates, respectively. Resistance for co-amoxiclav was found to be 27-47%, and for cefotaxime/ceftazidime and piperacillin/tazobactam around 10% (PHE, 2016a; PHW, 2015; HPS, 2014), through the situation for co-amoxiclav is confused by breakpoint changes.

Resistance to nitrofurantoin *in E. coli* occurs mainly by mutations in the chromosomal *nfsA* or *nfsB* genes encoding oxygen-intensive nitroreductases, which are involved in converting the drug into toxic intermediate compounds (Sandegren *et al.*, 2008). Recently, Ho *et al.* (2015) reported that the plasmid-mediated *OqxAB* efflux gene also can contribute to nitrofurantoin resistance in *E. coli* (Ho *et al.*, 2015). In general *E. coli* isolates show very low rates of resistance to nitrofurantoin *c.* 3% both in the UK (PHE, 2016a; PHW, 2015; HPS, 2014) and European countries (Kahlmeter, 2003; Kahlmeter & Poulsen, 2012).

Fluoroquinolones (in the UK, not elsewhere) and aminoglycosides are mostly reserved to treat hospital-acquired and complicated UTIs. High-level resistance to fluoroquinolones is generally mediated by mutations that alter the drug target and

mutations that affect permeation of the drug to its target or efflux (Jacoby, 2005). The mutation conferring high-level resistance occur in *gyrA* gene encoding DNA gyrase and *parC* gene encoding DNA topoisomerase IV. Plasmids that protect cells from the lethal effect of quinolones can also contribute to low level resistance (Hooper, 2001; Jacoby, 2005).

Aminoglycoside resistance occurs through several mechanisms that can coexist simultaneously in the same cell (Alekshun & Levy, 2007; Houghton *et al.*, 2010). These include (i) modification of the target by mutation of the 16s RNA (streptomycin only) or ribosomal proteins (Shaw *et al.*, 1993), (ii) methylation of 16s RNA (Galimand *et al.*, 2003; Doi & Arakawa, 2007), (iii) modification of outer membrane permeability or diminished inner membrane transport (Magnet & Blanchard, 2005), (iv) up-regulation of efflux pumps or (v) the most common mechanism in clinical strains- by enzymatic inactivation of the antibiotic molecules (Shaw *et al.*, 1993).

Among *E. coli* isolates resistance rates for fluoroquinolones (e.g. ciprofloxacin) and gentamicin have remained relatively unchanged at 15% and 10%, respectively from the last 4 years in the UK (PHE, 2016a; PHW, 2015; HPS, 2014).

The majority of Enterobacteriaceae, including ESBL producers, remain susceptible to carbapenems, and these agents are preferred as empiric therapy for serious Enterobacteriaceae infections, including urosepsis, in settings where ESBL producers are frequent; elsewhere effort is placed in conserving their utility. Carbapenem resistance, although rare, appears to be increasing e.g. through the spread of KPC, OXA-48 and NDM-1 enzymes (Paterson, 2006; Kumarasamy *et al.*, 2010) becoming a major public health problem. Better antibiotic stewardship and infection control are needed to slow and prevent the further spread of ESBLs, carbapenemases and other forms of resistance in Enterobacteriaceae throughout the world.

1.8 Diagnostics

Laboratory investigation of urine samples in the UK commonly relies on i) Triage on a screening system; ii) Culture on chromogenic agar; iii) Disc testing by BSAC (British Society for Antimicrobial Chemotherapy) or EUCAST (European Committee on Antimicrobial Susceptibility Testing) methodology. In recent years the diagnostic technology for urinary tract infections has improved significantly, with moves to mechanize traditional manual methods and to adopt fully automated systems. The pressure to increase laboratory efficiency, reduce costs, and allow clinicians to optimize therapy earlier prompts use of rapid and innovative technologies. Although microscopy and quantitative culture are still the gold standard, non-culture techniques with urine analyzers are increasingly used as the first triage to predict the presence of infections.

Public Health England proposed a diagnostic algorithm for better management of UTI in adults in primary care (PHE, 2014). The guideline recommend empirical treatment without routine culture for patients with uncomplicated UTIs with more than three clinical symptoms (i.e. any three or more of dysuria, urgency, polyuria, haematuria, frequency or suprapubic tenderness) without vaginal discharge or irritation. The diagnosis of mild UTI with fewer than two clinical symptoms requires collecting a urine specimen and performing a point-of-care dip-stick test to seek nitrites and leucocytes as UTI markers (see Section 1.8.3). If the results are both negative the GP should consider other diagnoses; negative nitrite and positive leucocyte or positive nitrite and negative leucocyte indicates a likely UTI, therefore the patient should be treated with the first-line antibiotic, and a simultaneous urine send to culture; if both markers are positive treatment with first-line agents is required.

1.8.1 Visual inspection

The primary urine analysis is based on the visual inspection of colour, turbidity and odour. The urine colour reflects the dehydration level, consumption of certain foods (e.g. beets, berries) or medicine, but may also change with infections requiring

treatment. Normal urine colour ranges from pale yellow to deep amber depending on the pigments present (urochrome and urobilin) and their concentration. A cloudy urine or one with unusual colour such as white can occur due to vaginal discharge or kidney stones (Bartley, 2008); deep red to brown generally indicates secretion of excess bilirubin into the urine potentially indicating presence of certain diseases including liver cancer or acute hepatitis (Fernandez & Flaxman, 1985); blue or blue green could be associated with benign hypercalcaemia or infection caused by *Pseudomonas* spp. (Chung *et al.*, 2008).

1.8.2 Classical microscopy investigation

Classical microscopy for the detection of urinary tract infection looks for red blood cells (RBCs), white blood cells (WBCs), epithelial cells, casts, crystals, and bacteria.

A microtitre tray with an inverted microscope or a disposable counting chamber are recommended for semi-quantitative routine analysis. Due to methodological limitations, the sensitivity for detecting bacteria in uncentrifuged, unstained urine is around 10^7 cfu/L (10^4 cfu/mL). Some studies have found that experienced workers can achieve better diagnostic precision if the urine is centrifuged and/or Gram-stained (Pezzlo, 1988), however this is time consuming for centralized microbiology laboratories, where large numbers of urines are submitted for analysis.

Significant pyuria of ≥10^4 WBC/mL in a freshly voided urine specimen or >5 WBC in a high-power microscope field of centrifuged urine correlates with bacteriuria and clinical symptoms to indicate UTI. Significant pyuria is present in 96% of symptomatic patients with bacteriuria of >10^5 cfu/mL, but only in <1% of asymptomatic patients (HPA, 2011.). Pyuria in the absence of bacteriuria (≥10^4 WBC/mL) may be the result of many factors, including the presence of a foreign body (urinary catheter, urinary stones or neoplasms) (Khamees, 2012), infection caused by fastidious bacteria difficult to cultivate on standard medium (Hooker *et al.*, 2014), prior antibiotic treatment reducing the bacterial count, renal tuberculosis especially among high-risk

patients (Kulchavenya *et al.*, 2013), vaginal secretion and chronic UTIs associated with relatively sterile urine (men).

Bacteriuria (>10^5 cfu/mL) in the absence of pyuria may indicate colonization of the lower urinary tract, contamination of the sample (Stansfeld, 1962), poor urine storage in routine laboratories or during transport to these laboratories.

Pathological findings in urine can also relate to acute or chronic inflammatory processes in the kidneys, elsewhere the urinary tract and to other diseases. In particular the detection of RBCs in urine (haematuria) may reflect pre-renal, renal or post-renal diseases, but also occurs in certain physiological conditions such as menstruation or following strenuous exercise. Casts (hyaline, cellular, granular) are cylindrical protein mouldings formed in renal tubules; their presence may indicate renal pathology such as pyelonephritis, glomerulonephritis, nephritis, tubular necrosis and nephrotic syndrome. Crystals are often associated with the formation of urinary tract calculi or acute hepatic injury but they may be also benign. Identification of epithelial casts is a useful indicator of the degree of contamination from the perineal region.

1.8.3 Rapid point-of-care screening

Chemical dipstick tests are widely used to recognize pathological changes in urine, and to infer UTI. Figure 4 shows an exemplar of result interpretation for a commercial dipstick test. Conclusion are based on the detection of the colour changes contingent on the presence of compounds, enzymes or cell types that ordinarily are absent from urine e.g. nitrite, leukocyte esterase, erythrocytes, protein (albumin), glucose, ketone bodies, bilirubin, urobilinogen and ascorbic acids; pH is also tested.

The presence of nitrite, which is the metabolic product of Enterobacterial nitrate reductase, indicates infections caused by Gram-negative rods, however it is not detected if the causative uropathogen is not nitrate-reducing (or if reduces nitrate to nitrogen gas) as with e.g., *Enterococcus* spp., *S. saprophyticus, Pseudomonas* spp., or *Acinetobacter* spp. Therefore the sensitivity of the method is variously reported as

from 75-85%, but specificity is higher at around 95% (Hooton & Stamm, 1997; Orenstein & Wong, 1999; ECLM, 2000).

Leucocyte esterases are detected on the basis of indoxyl esterase activity. They arise from the neutrophil granulocytes and macrophages, which enter the urine both in UTIs and in non-infectious renal disease. The sensitivity for UTI detection ranges from 75-96%, and specificity from 94-98% (Williams *et al.*, 2010).

Other tests on the dipstick are not primarily related to the infection. Detection of RBCs or their haemoglobin relies on pseudoperoxidase activity from the haem moiety and corresponds with renal diseases. It can be found in patients with muscle necrosis, alcohol and cocaine abuse, polymyosities. The sensitivity for RBC detection is in a range 70-80%, but specificity is lower due to rapid RBC lysis.

Proteinuria reflects renal, prostatic or bladder diseases and vaginal discharge. The sensitivity is 90-95% for clinical proteinuria (Schmiemann *et al.*, 2010). Urine glucose measurement in usually combined with a blood glucose test to reveal diabetes. Ketone bodies appear in urine in diabetic acidosis, strenuous exercise, fasting and enteric inflammation. They are used as a marker to classify of treat specific patient populations. Bile pigments such as urobilinogen and bilirubin are useful in differentiating icteric patients or in detecting alcoholic liver disease.

Urinary pH varies between 5 and 9. Urine usually is acidic in the morning in adults whilst it is often alkaline in children. Measurements of urine pH are needed for the diagnosis of acid-base disturbances, renal tubular acidosis, and renal stone disease or during the elimination of specific drugs.

Dipstick tests are quick, easy to perform, and can be carried out in primary care, giving an immediate result including detection of relevant infection. However, the leucocyte esterase and nitrite tests are less reliable in diabetic and elderly individuals (Ipe *et al.*, 2013). Moreover, both markers have poor positive and negative predictive values for asymptomatic bacteriuria. The presence of bacteria alone in urine specimen may not necessarily be of clinical significance. It may indicate colonization or contamination in the absence of pyuria (Ipe *et al.*, 2013).

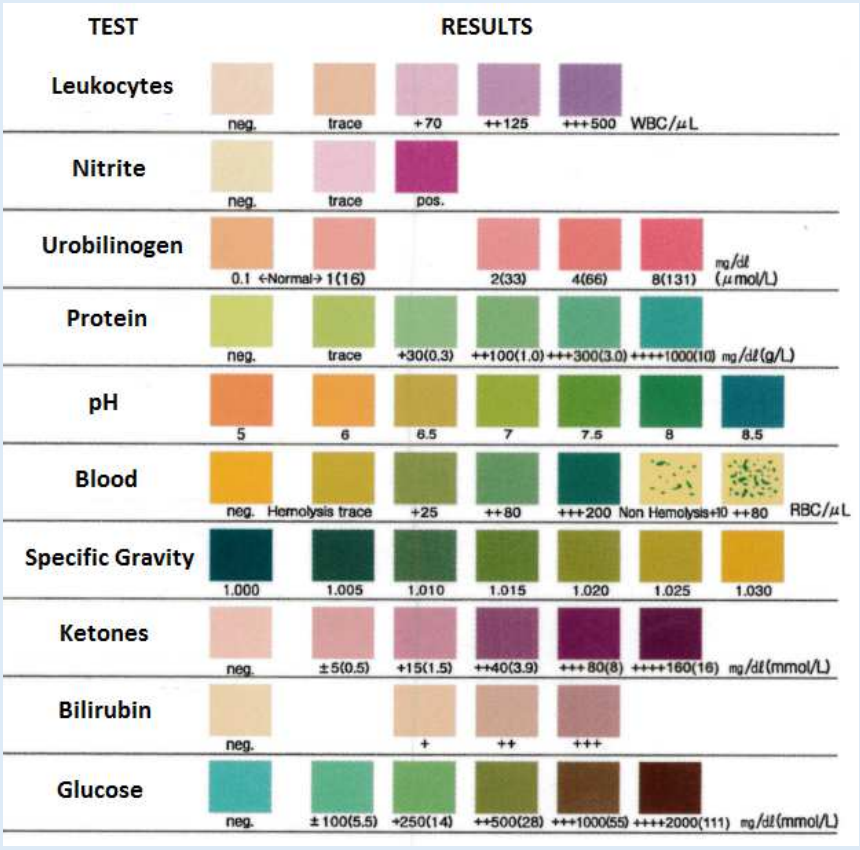

Figure 4. Chemical dipstick test screening for urinalysis.

The dipstick consists of small pads containing reagents which react with compounds present in urine producing characteristic colours. The results are available after a few minutes as qualitative (positive or negative) or quantitative values reported as (1+, 2+, 3+, 4+) or milligrams/decilitre.

1.8.4 Machine-based screening

Rapid semi-automated urine analysers are an alternative to manual laboratory screening methods. They are particularly useful to exclude non-infected patients, reducing unnecessary manual work on these specimens. They also promote standardisation. The systems require a small volume of urine and abolish the problems associated with microscopic examination of urine sediment which needs to be a centrifuge before analysis. As with manual microscopy they seek RBCs, WBCs, crystals, casts, epithelial cells, mucus, bacteria and yeasts. Currently-available

automated screening systems include those based on (i) flow-cell digital imaging, (ii) fluorescence flow cytometry, (iii) sediment analysis.

Flow-cell digital imaging

The iQ®200 (Iris Diagnostics, Chatsworth, CA, USA) analyser (Figure 5) is the only currently-available automated microscopic system to screen urine specimens based on flow cell digital images with automatic particle-recognition software. Particles are classified-on the basis of texture, contrast, shape and size-into 12 categories (e.g. as WBC, RBC or bacteria). Quantitative results are presented as number of particles of each sort/µL, /high power field, /low power field. Throughput is 60 samples/hour. The reliable detection limit is 6 particles/ µL (Alves *et al.*, 2005).

Stürenburg *et al*. examined 963 urines using the iQ200 system against a culture method to assess cut-off values. Using different threshold combinations of indicators (e.g. bacteria, leucocytes and all small particles) he achieved sensitivity at 95% for the detecting of UTI, reducing the proportion of urines needing culture by 30.4-35.9% (Stürenburg *et al.*, 2014). In other studies (Linko *et al.*, 2006; Alves *et al.*, 2005; Čabarkapa *et al*., 2009) the iQ200 showed good performance to reliably count RBC, WBC, squamous epithelial cells, and to detect bacteria and renal elements.

Figure 5. iQ®200 Elite urine screening analyzer.

Specimens are hydrodynamically focused between two layers of suspending fluid. The particles are viewed through the objective lens of a microscope connected with a camera. The analyser captures 500 frames/samples from the planar flow of urine. The software displays the images for visual confirmation.

Sediment analyser

The SediMax (Menarini Diagnostics, Florence, Italy) shown in Figure 6, is walk-away microscopic sediment analyser for urine screening. The system depends upon digital imaging and automatic particle-recognition software enable to distinguish urine components (human cells, cast, crystal) and pathogens within whole fields of view. Quantification of these particles is either as number/µL or /high power field. The identified particles can be seen on the screen as black and white images obtained by microscopy. Throughput is 100 samples/hour.

The performance varies depending on the bacteria and leukocyte count cut-offs applied. Sterry-Blunt *et al.* (2015) reported that analyser is not suitable to screen routine urines prior to culture due to a low negative predictive value (87.5%) (Sterry-Blunt *et al.*, 2015) but other studies showed that the system could be reliably applied to urine screening (Tessari *et al.*, 2015; Falbo *et al.*, 2012) decreasing (as with an iQ®200 system) the number of unnecessary urine cultures performed and thereby reducing both costs and workload. Overall, compared to manual microscopy the instrument performed well for detecting bacteriuria, RBCs, WBCs, yeast cells, but not as well for pathological casts and particularly squamous epithelial cells (Bogaert *et al.*, 2016).

Figure 6. SediMax urine screening analyzer.

The sediment is analyzed by a bright field microscope and with a digital camera to capture and categorize 15 particle types based upon size and shape, using image processing software.

Fluorescence flow cytometry

This technology combines a diode laser with hydrodynamic focusing conductometry for bacterial detection and counting. The first commercially available system was UF-100 (Sysmex Corporation, Kobe, Japan) using electrical impedance to measure forward light scatter as a marker of human or bacterial cell, size and length. The system was updated to the UF-1000i (Figure 7) with a separate 'BACT' analytic channel measuring fluorescent light from bacterial DNA stained with phenathridine dye. Particulate components in the urine are discriminated by size and stain sensitivity, and the results are displayed as scattergrams.

The sensitivity and specificity of the system vary depending on the parameters and cut-offs employed. Wang *et al.* (2009) and Manoni *et al.* (2013) reported a sensitivity of 97%, and specificity in a range 79-94% when the definition of a negative urine culture was $<10^5$ cfu/mL (Wang *et al.*, 2013b; Manoni *et al.*, 2009). In another study, the sensitivity was 95%, and specificity 85% for 'negative' urine culture with $<10^4$ cfu/mL (De Rosa *et al.*, 2010). It was suggested that cut-off criteria should be chosen for an analyser to balance the levels of sensitivity and specificity required according to a local assessment.

Many studies show that the pre-screening with the Sysmex UF-1000i can potentially reduce the culture-negative samples put through full testing by more than 40% (van der Zwet *et al.*, 2010; Broeren *et al.*, 2011). Throughput is higher compared to the iQ®200 flow cell imaging system at 100 samples/hour.

Figure 7. UF-1000i urine screening analyzer.

The laser-beam irradiates a fluorescein-stained urine sample that produce signals as forward-scattering light, side-scattering light and side-fluorescence signal. These are converted into optoelectronic signals, enabling component identification, counting and analysis.

1.8.5 Culture-based methods

None of the automated screening analyser identify species of bacteria present in a urine and test their antibiotic susceptibility. Culture thus remains the "gold standard" for investigation of these aspects and may be performed on all urines or only on those still considered as "possibly infected" following triage with a screening analyser as above.

Quantitative urine culture is commonly performed using calibrated loops, usually on unselective media including blood agar, MacConkey, or Cysteine Lactose Electrolyte Deficient (CLED) agar or the selective chromogenic agar where the colony

colour varies with the organism species. These methods require 24-72 h for bacterial identification, with subsequent susceptibility testing and refinment of whether empirical treatment was started. Despite near-universal use culture has inadequate sensitivity and specificity to define 'significant' bacteriuria in all the diversity of clinical scenarios (Ipe *et al.*, 2013).

Approximately one-fourth of patients presenting with symptoms suggesting acute uncomplicated UTI yield no bacterial growth on urine culture or give counts between 10^2 - 10^4 cfu/mL (Gallagher *et al*., 1965). Acute pyelonephritis can occur with lower bacterial counts than the classical 10^5 cfu/mL, especially among some groups of patients (MacDonald *et al*., 1957) e.g. children, pregnant women, and elderly populations with comorbid illnesses. These points underline the need for new, more sensitive diagnostic methods that could detect bacteriuria with lower bacteria counts.

1.8.6 Classical bacterial identification

The bacteria grown from the urine and then are identified. Traditional methods for identification rely on cell morphology, staining and chromogenic reactions in biochemical tests. Technologies include:

Chromogenic media

Identification (combined with the initial isolates) is based on colony pigmentation or colour changes to chromogenic agar due to bacterial interactions with specific dyes or chemicals in the growth media, as shown in Figure 8. Urine is plated directly on the agar using calibrated loop, without prior culture, to assess the present of uropathogens. Chromogenic media can distinguish Gram-negative bacteria including *E. coli*, 'other Coliforms', Proteeae, *Pseudomonas* spp., and Gram-positive pathogens e.g. *Enterococcus* spp., *Streptococcus* spp., *Staphylococcus* spp. and *S. saprophyticus.*

E. coli and *S. saprophyticus* both produce the enzyme β-glucuronidase, which attacks a β-glucuronide chromogenic substrate, and consequently grow as distinct

pink to reddish colonies. Although both species give the same colour*, E. coli* colonies are bigger and darker than *S. saprophyticus.* Others coliform species such as *Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp., and *Serratia* spp. produce the enzyme β-glucosidase that attacks a β-glucoside chromogenic substrate to give deep metallic blue colonies.

Enterococcus spp. produces the same enzyme β-glucosidase resulting in growth as small turquoise blue colonies. Tryphan deaminase activity produce a brown halo around *Proteus* spp., *Morganella* spp., and *Providencia* spp. colonies. *S. aureus* and *Candida* spp. produce their natural pigments, resulting in grow as small white colonies. *Pseudomonas* spp. grows as cream, translucent colonies.

Distinguishing among 'other coliforms' (*Enterobacter* spp., *Klebsiella* spp., *Citrobacter* spp., *Serratia* spp.) is not achieved, therefore further identification to genus level is performed, if desired using other systems (e.g. Vitek, MALDI-TOF).

Chromogenic media are produced by different companies. The commonest include Brillance UTI or Brillance UTI Clarity agar (Oxoid, Basingstoke, UK), ChromID™ (bioMerieux Clinical Diagnostics, Marcy l'Etoile, France) and CHROMagar Orientation™ (Biomed Diagnostics, Oregon USA).

Samra *et al.* (1998) evaluated the CHROMagar Orientation plate using 900 urine specimens from hospital patients. The study showed that orientation media detected all uropathogens (Gram-negative bacilli, streptococci, staphylococci and yeast) grown on reference media (5% sheep blood and MacConkey agar), however it failed to distinguish *Klebsiella, Enterobacter* and *Citrobacter* isolates owing to their similar pigmentation. Otherwise, CHROMagar Orientation plates were useful for primary identification of uropathogens (Samra *et al.*, 1998).

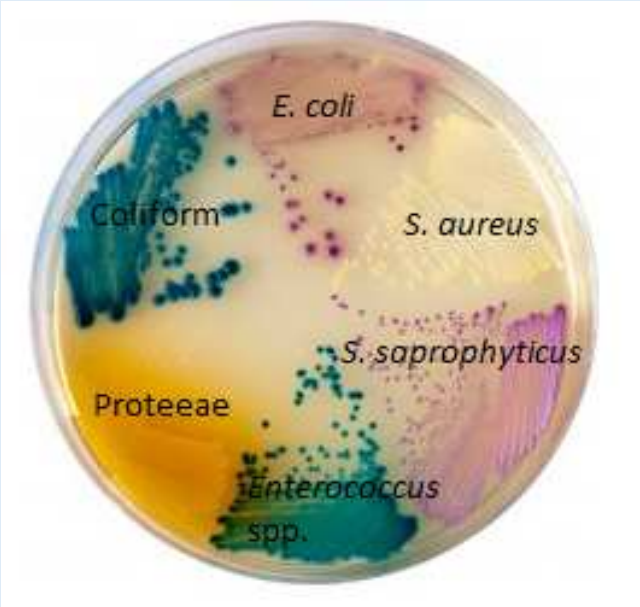

Figure 8. Phenotypic identification of bacteria using CHROMagar Orientation™ media (Biomed Diagnostics).

API ID Strips

Until recently API ID strips were the standard identification method for bacteria in most clinical laboratories. Urine is first plated on the media (e.g. CLED or MacConkey). Colonies that grow are resuspended in a sterile water or saline to the density of a 0.5-4 McFarland and then transferred into microtubes (cupules) of the API plastic strip (Figure 9). Identification is based on colour changes due to enzymatic activity or the assimilation/fermentation of sugars using miniaturized biochemical tests. When carbohydrates are fermented, the pH within the microtubes changed as shown by a pH indicator. In assimilation tests (e.g. with citrate) the test shows if the bacteria are able to utilize the substrate as a carbon and energy source. After overnight incubation results are read, converted to a profile number and the bacterial identification is determinated by reference to an online database.

Depending on the pathogen, there are different API strips available (bioMerieux Clinical Diagnostics). These include the API 20 NE for identification of oxidase-positive non-fastidious Gram-negative bacilli, API 20 E for *Enterobacteriaceae* and oxidase-

negative, non-fastidious other Gram-negative bacilli, API 20 Strep for streptococci and enterococci groups, API ID32 Staph for staphylococci and API NH for Gram-negative cocci.

The API identification system was introduced to the clinical laboratories in 1970s and gradually replaced manual biochemical tests done in tubes. In 1990s O'Hara *et al.* revaluated the API 20E strip versus conventional biochemical tests for the identification of 291 *Enterobacteriaceae* isolates (O'Hara *et al.*, 1992). They achieved 78.7% accuracy of identification after 24h incubation period. This was significantly lower than earlier evaluations (Aldridge *et al.*, 1978; Butler *et al.*, 1975; Swanson & Collins, 1980) but rose to 95.2% after 48h incubation. Despite this lower identification precision the study considered that API 20 E remained a reliable identification system for *Enterobacteriaceae.* However, the method have several drawbacks including limited databases that do not contain a new species (Janda & Abbott, 2002) leading to unreliable identification, relatively long turnaround (24-48h), low capacity and lack of automation. These limitations mean that the UK system has gradually been replaced by new rapid methods (e.g. Vitek and MALDI-TOF).

Tests	Active ingredients	Reaction/Enzymes	Results	
			Negative	Positive
ONPG	2-nitrophenyl-ßD-galactopyranoside	β-galactosidase (Ortho NitroPhenyl-βDGalactopyranosidase)	colourless	yellow
ADH	L-arginine	Arginine dihydrolase	yellow	red/orange
LDC	L-lysine	Lysine decarboxylase	yellow	red/orange
ODC	L-ornithine	Ornithine decarboxylase	yellow	red/orange
CIT	trisodium citrate	CITrate utilization	pale green / yellow	blue-green / blue
H_2S	sodium thiosulphate	H_2S production	colorless / greyish	black deposit / thin line
URE	urea	UREase	yellow	red/orange
TDA	L-tryptophan	Tryptophan deaminase	TDA /immediate	
			yellow	reddish/brown
IND	L-tryptophan	INDole production	James/ immediate	
			colourless pale green/yellow	pink
VP	sodium pyruvate	Acetoin production	VP1 + VP2 10 min	
			colourless	pink/red (after addition of reagent)
GEL	gelatin (bovine origin)	GELatinase	no diffusion	diffusion of black pigment
GLU	D-glucose	Fermentation/oxidation	blue/blue-green	yellow/greyish yellow
MAN	D-mannitol	Fermentation/oxidation	blue/blue-green	yellow
INO	inositol	Fermentation/oxidation	blue/blue-green	yellow
SOR	D-sorbitol	Fermentation/oxidation	blue/blue-green	yellow
RHA	L-rhamnose	Fermentation/oxidation	blue/blue-green	yellow
SAC	D-sucrose	Fermentation/oxidation	blue/blue-green	yellow
MEL	D-melibiose	Fermentation/oxidation	blue/blue-green	yellow
AMY	amygdalin	Fermentation/oxidation	blue/blue-green	yellow
ARA	L-arabinose	Fermentation/oxidation	blue/blue-green	yellow

Figure 9. Bacterial identification using the API ID Strip system.

The API 20 E was read after 24h at 37°C and results are illustrated for an *E. coli.*

Matrix Assisted Laser Desorption Ionization Time-Of-Flight (MALDI-TOF)

Mass spectrometry has recently been adopted widely in diagnostic microbiology laboratories for bacterial and yeast species identification from primary cultures (Bizzini *et al.*, 2010; Marklein *et al.*, 2009; van Veen *et al.*, 2010). It has also been investigated for use directly from clinical samples (Ferreira *et al.*, 2011; Wang *et al.*, 2014a; Rossello *et al.*, 2014; Hong *et al.*, 2014).

The MALDI-TOF instrument consists of three principal components: i) an ionization chamber, within which the laser-based vaporization of the specimen takes place, ii) a time of flight mass analyzer, and iii) a particle detector (see Figure 10). Biopolymer molecules (e.g. DNA, protein, peptides), normally present in the condensed phase, are converted into intact ionized molecules in the gas phase. There ions are then separated based on their molecular weight after migration in an electric field. Each molecule detected is characterized by: its molecular mass (m), its charge (z), its mass/charge ratio (m/z), and the relative intensity of the signal (Carbonnelle *et al.*, 2012). The system generates a unique, specimen specific mass spectral protein fingerprint from a cell extract of the tested microorganism, and compare this profile to a database of reference spectra.

Currently, there are two commercially available MALDI-TOF systems. These are the VITEK MS (bioMérieux Clinical Diagnostics) and MALDI Biotyper (Brüker, Daltonics GmbH). Both machines demonstrated similar efficiency, with a high accuracy to genus/species-level identification and workflow robustness (Bilecen *et al.*, 2015). Both provide results in less than 1 hour. The major difference is the number of taxa in the reference database. The VITEK MS system contains >25,000 spectra covering 586 species, consisting of 508 bacteria and 78 fungi, while Brüker Biotyper contains >80,000 spectra covering 2048 species and 385 genera (Bilecen *et al.*, 2015). Limitation are seen in relation to some groups: neither can distinguish *E. coli* from *Shigella* spp. and nor can they differentiate within the *Acinetobacter baumannii* complex.

Detection of antibiotic resistance profiles by MALDI-TOF is challenging but remains under evaluation. Recent studies have investigated the use of MALDI-TOF to discriminate extended-spectrum-β-lactamase (ESBL) or metallo-β-lactamase (MBL)-

producing and -non-producing strains of Enterobacteriaceae (Schaumann *et al.*, 2012; Hoyos-Mallecot *et al.*, 2014a). The approach generates different molecular mass profiles when the central β-lactam ring of a carbapenem or extended-spectrum cephalosporin is opened by enzymatic hydrolysis. If bacteria are incubated with β-lactams, these changes can be used to detect β-lactamase activity.

These β-lactamase assays use commercially-available software (Brüker Daltonics GmbH) but are still under evaluation and need to be validated in the clinical laboratories.

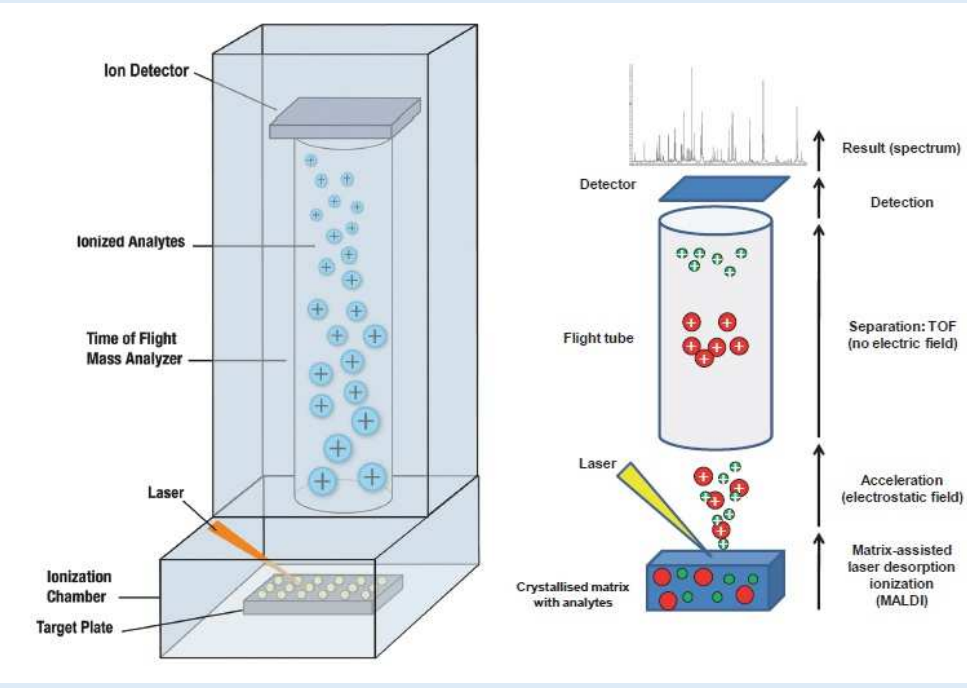

Figure 10. The workflow of MALDI-TOF MS (Brüker Daltonics GmbH).

There are several automated systems that combine both classical identification and antibiotic susceptibility testing (i.e. Vitek, MicroScan WalkAway, BD Phenix and Sensititre ARIS 2x). These are included in the section 1.8.7.2 (Automated systems for both bacterial identification and susceptibility testing).

1.8.7 Classical antimicrobial susceptibility testing

The main purposes of in vitro susceptibility testing are i) to guide treatment of the immediate patients, ii) to assess appropriate empirical therapy, based on the local resistance epidemiology and iii) to compare resistance rates to new and established agents over time. Current systems for susceptibility testing from urine are outlined below. The interpretation of results is based on measurement of the zone diameter or MIC (minimal inhibitory concentration) of each antibiotic. Results are classified into one of three interpretative categories: susceptible, intermediate, or resistant using the criteria provide by EUCAST (European Committee on Antimicrobial Susceptibility Testing) or CLSI (Clinical and Laboratory Standards Institute). Larger zones in disc tests correlate with lower MICs of antibiotics, including susceptibility, and small zones or the absence of zones reflect high MICs and resistance. For most antibiotics there is good correlation between log MIC and zone diameter in disc tests.

1.8.7.1 Manual antibiotics susceptibility methods

Broth and agar dilution

In dilution methods a standardized inoculum of bacterial cells are tested for their ability to produce visible growth on agar plates ("agar dilution") or in broth ("broth dilution") containing geometric dilutions (0.25, 0.5, 1, 2, 4, 8, 16 etc. mg/L) of the antimicrobial. Growth is assessed after incubation for a defined period of time usually 16–20 h and MIC results are read.

Antimicrobial gradient strips (E-test)

A reagent strip containing a gradients of an antibiotics is placed onto the surface of an agar plate inoculated with the test bacteria. The strip releases the antimicrobial into the agar and forms a stable gradient. The MIC value is read from the scale as mg/L or µg/mL where the ellipse edge intersects the strip (Figure 11A).

Disc Diffusion

This method, the most widely used in routine clinical laboratories in the UK, is based on measurement of growth zone inhibitions. Discs containing antibiotics are placed onto agar inoculated with bacteria to a standardized density (Figure 11B). The plate is examined after 18-24 h of incubation and the zones measured.

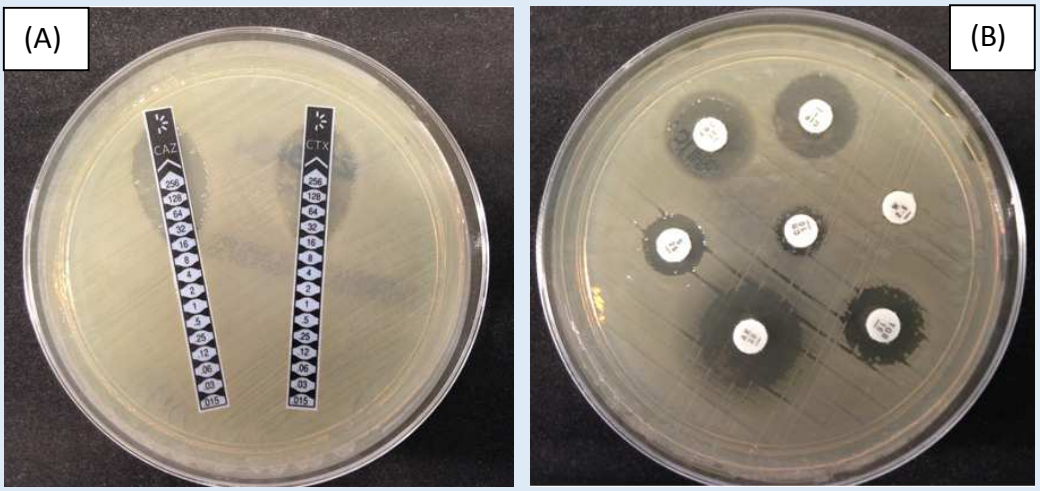

Figure 11. Antibiotics susceptibility testing using gradient strips and a disc diffusion method.
Both tests were performed on Mueller-Hinton agar with a bacterial density equal to a 0.5 McFarland. Gradient strips (Oxoid) (A) show MIC results for ceftazidime and cefotaxime. Disc diffusion test (B) with discs containing the following antibiotics: co-amoxiclav (AMC), ciprofloxacin (CIP), streptomycin (S), gentamicin (CN), trimethoprim (W), amikacin (AK), tobramycin (TOB) (Oxoid)

Biochemical tests

Biochemical and phenotypic tests are widely used to detect specific β-lactamase types. Examples include various double disc synergy and antagonism test for ESBL and AmpC enzymes. Detection of carbapenemase-producing Enterobacteriaceae is possible using the recently released commercially RAPIDEC CARBA NP kit and its variations (Rapid CARB Screen and the Carba NP test) (bioMérieux Clinical Diagnostics). Results are obtained within 30 min to 2 hours using colonies recovered from the culture plate. The test is based on detection of hydrolysis of the β-lactam

ring of a carbapenem molecule (imipenem), which generates acidity, changing the colour of the phenol red pH indicator. The principle is that of the long-established acidimetric test. The sensitivity and specificity of the assay is 97.8% (except OXA-48) compared to molecular methods.

1.8.7.2 Automated systems for both bacterial identification and susceptibility testing

The use of automated systems enables standardization of testing reading and results; it also speeds-up susceptibility testing. The aim is to classify bacteria as susceptible, intermediate or resistant against particular antibiotics. There are four commonly used systems available:

Vitek®

This is a fluorimetric test with biochemical substrates for bacterial growth during an abbreviated incubation period in the microwells of thin plastic reagent cards with microlitre quantities of antibiotics and test media (see Figure 12A). The system requires cultivated bacteria grown on specific media. The technology is available in three formats: Vitek 2 Compact, Vitek 2 and Vitek 2 XL (bioMérieux Clinical Diagnostics) differing in capacity and automation. All three systems accommodate the same reagent cards and therse are incubated and interpreted automatically, yielding identification for Gram-negative fermenting and non-fermenting bacilli, Gram-positive cocci and spore- and non-spore forming bacilli, yeasts along with susceptibility testing. The results are available within 8-10 hours.

The reagent cards have 64 wells each containing an individual test substrate or antibiotic dilution. Substrates measure various metabolic activities such as acidification, alkalization, enzymatic hydrolysis and growth in the presence of inhibitory substances. Each card has a pre-inserted transfer tube used for inoculation.

Many studies have evaluated the Vitek system for rapid identification and susceptibility testing against different types of clinical isolates (Ling *et al.*, 2001; Joyanes *et al.*, 2001). In most studies the technology gave reliable and reproducible results, with >95% of isolates correctly identified to species level.

MicroScan WalkAway system

The technology compromises with large self-contained incubator/reader for analyzing 40–96 microdilution trays (Figure 12B) for identification of both Gram-negative and Gram-positive bacteria and susceptibility testing. The instrument incubates the trays which contain substrates for around 16-18 h. Identification of the bacteria is based on periodically measuring colour change or increases in turbidity. For susceptibility testing the system reads the MIC essentially using conventional micro-titration trays containing antibiotic dilution series. The use of fluorogenic growth substrates give results readable in 3.5–7 h for Gram-negative bacteria; alternatively turbidimetric end points for Gram-positive and -negative bacteria are available in 4.5–18 h (Jorgensen & Ferraro, 2009). Rhoads *et al.* (1995) compared the MicroScan WalkAway system (Beckman Counter, Brea, CA, USA) with Vitek (bioMérieux Clinical Diagnostics) for identification of Gram-negative bacteria from urine isolates. The authors found 97.4% agreement between these two methods (Rhoads *et al.*, 1995). In another study, the system was compared against reference molecular methods for detection of ESBL producers among Enterobacteriaceae, finding sensitivity and specificity at 83.5% and 72.9%, respectively (Wiegand *et al.*, 2007).

BD Phoenix system

The Phenix technology (BD Diagnostics, Sparks MD, USA) compromises an incubator/reader with the capacity to process 100 test panels for pathogen identification and susceptibility testing. Different panels are used for Enterobacteriaceae, non-Enterobacteriaceae and Gram-positive bacteria including staphylococci, enterococci and streptococci and also for Gram-positive bacilli.

The panel contains 136 micro-wells, including 51 wells for identification and 85 wells for susceptibility testing including one growth control. Panels are available separately or with combining of identification and susceptibility.

Panels are inoculated manually and then incubated into the instrument. The system monitors each panel every 20 min up to 16 h, if necessary, using both turbidometric and colorimetric (oxidation, fermentation, hydrolysis indicator) growth

detection. Additionally, the instrument utilizes chromogenic and fluorogenic substrates for pathogen identification (Figure 12C). MIC results are generated in 6–16 h. There are many studies evaluating the use of the Phoenix system for pathogen identification and susceptibility testing (Carroll *et al.*, 2006; Stefaniuk *et al.*, 2003; Snyder *et al.*, 2008); the platform provides reliable results for most organism-antimicrobial agent combination.

Sensititre ARIS 2X system

This is a bench-top incubating and reading system with a 64-panel capacity for both identification and susceptibility testing. The test panel are standard 96-well microdilution plates which can be inoculated with a Sensititre auto inoculator. The system is based on fluorometic detection of bacterial growth (Figure 12D). Test panels are available for the most common aerobic Gram-positive and Gram-negative bacteria. Presumptive identification of Gram-negative pathogens can be obtained within 5 h, however comprehensive identification to species level is only available after 18 h. Standard susceptibility MIC plates include those for Gram-positive and Gram-negative (with an ESBL confirmatory test bacteria), anaerobes, *Campylobacter*, and fastidious pathogens (*S. pneumoniae, Haemophilus influenzae*). A few studies have compared the Sensititre ARIS 2x system (Trek Diagnostic Systems, Cleveland, OH, USA) against Vitek (bioMérieux Clinical Diagnostics) or BD Phoenix (BD Diagnostics) as a reference methods (Fritsche *et al.*, 2011; Dickenson & Chapin, 2006) finding that the platform can be used to accurately identify and determinate susceptible including fastidious pathogens.

Figure 12. Commercially available systems used for pathogen identification and antibiotic susceptibility testing.

(A) Vitek 2 Compact (bioMerieux Clinical Diagnostics)
(B) MicroScan WalkAway (Beckman counter)
(C) BD Phoenix (BD Diagnostics)
(D) Sensititre ARIS 2X (Trek Diagnostic Systems)

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1.8.8 New developmental methods to improve diagnostics

Global concern about antibiotic resistance, the lack of new classes of antibiotics and increased hospital admissions for infections, including complicated UTIs, mean that there is a need for rapid, sensitive and specific methods for both pathogen identification and resistance profiling directly from clinical specimens, including urines. Possible approaches include those based upon PCR, sequencing, and rapid phenotypic testing.

Novel tests performed directly on clinical samples, without culture have the potential to significantly shorten the time required for microbial identification and to select appropriate therapy. This would improve care of individual patients and reduce unnecessaily prolonged use of broad-spectrum antibiotics, which presently must be given until a pathogen is grown and characterized by conventional methods, which typically take a total of 48-72 h.

1.8.8.1 PCR-based assays

Conventional PCR, with subsequent gel electrophoresis, was progressively replaced by multiplex PCR and quantitative real-time PCR. Multiplex PCR uses multiple primers to detect several genes simultaneously and therefore requires specific primers that do not self-and cross-react. The real-time technology uses fluorescence sequence-specific probes or non-specific fluorescent dyes that intercalate with double-stranded DNA, allowing monitoring of the amplified product. The results are displayed as fluorescence intensity of the desired product against the number of amplified cycles (Ct curve analysis) and/or a melting temperature graph (melting curve analysis). Combining multiplex PCR and real-time analysis gave an opportunity to detect multiple targets in a single reaction in a short turnaround time.

Within these main PCR types there are many other variations that can be used for rapid detection of pathogens and antibiotic susceptibility testing. These include (i) digital PCR, where a single DNA molecule is isolated by dilution and individually amplified by PCR (Vogelstein & Kinzler, 1999; Kelley *et al.*, 2013; Roberts *et al.*, 2013), (ii) asymmetric PCR later modified to Linear-After-The-Exponential PCR (LATE-PCR),

where one strand of the target DNA is amplified with a limiting amount of primer (Carver-Brown *et al.*, 2012; Rice *et al.*, 2013) and (iii) nested PCR, where two sets of primers are used, one pair to generate DNA template and the second pair to amplify a specific product from the first step (Gómez-Couso *et al.*, 2004).

Several commercially available tests have been developed variously targeting bloodstream infections, respiratory pathogens, sexually-transmitted diseases or enteric infections. For example, bloodstream pathogens may be identified, without culture, using the following tests Magicplex Sepsis Test (Seegene, Seoul, Korea), SepsiTest (Molzym, Bremen, Germany), SeptiFast (Roche, Basel, Switzerland), Bacteraemia Panel (Ausdiagnostics, Sydney, Australia) and Sepsis Microbial DNA qPCR array (Qiagen, Hilden, Germany). These kits seek the commonest bacteria causing bloodstream infection, including Enterobacteriaceae and *Pseudomonas* spp., also Gram-positive cocci including staphylococci, streptococci and enterococci as well as *Candida* yeasts.

In-house PCR-based methods for the diagnosis of UTIs have been described (van der Zee *et al.*, 2016; Hansen *et al.* 2013; Shigemura *et al.*, 2005; Lehmann *et al.*, 2011). These sought the commonest Gram-positive and Gram-negative uropathogens including *E. coli, Klebsiella* spp., *Citrobacter* spp., *Enterobacter* spp., *Proteus* spp., *Pseudomonas* spp., *Enterococcus* spp., *Streptocossus* spp. and *Staphylococcus* spp.

Van der Zee *et al.* (2016) evaluated two multiplex real-time PCR assays for targeted common uropathogens, and simultaneously performed 16S PCR to identify all microorganisms in there urine specimens. The authors showed that the concordance between the real-time approach and culture was 98%, with PCR more sensitive than culture. Hansen *et al*. (2013) demonstrated that the multi-probe 16S rDNA-based real-time PCR has a sensitivity 97% and specificity 80% and provide results within 4 h.

Molecular assays for antibiotic resistance gene detection also are accessible for clinical microbiology laboratories, through largely for use on nasal or rectal swabs to seek carriers of resistant strains so as to implement infection control rather than to guide therapy (through those for TB are exception). Available commercial PCR assays seek the presence of (i) *mecA* and *mecC* genes that confer methicillin resistance in *S. aureus* (e.g. Cepheid Xpert/Gene Expert, Cepheid Sunnyvale, CA, USA; BD GeneOhm

MRSA Becton Dickenson, Heidelberg, Germany; LightCycler® MRSA Advanced Test, Roche)*;* (ii) *vanA, vanB* and *vanC* genes that confer glycopeptide resistance (e.g. Xpert/Gene Expert, Cepheid; LightCycler® VRE Detection Kit, Roche); (iii) mutations in *rpoB* that confer resistance to rifampicin or those in the *katG* and *inhA* genes that confer resistance to isoniazid (e.g. Xpert MTB/RIF, Cepheid, RealTime MDR TB Abbott Molecular, Illinois, USA). In recent years the growing spread of β-lactamases among Enterobacteriaceae has lead to development of several commercial PCR-based assays seeking the coding genes for extended-spectrum β-lactamase (*bla*$_{\text{CTX-M}}$), *ampC* (mostly *bla*$_{\text{CMY}}$), penicillinases (e.g. *bla*$_{\text{TEM,}}$ *bla*$_{\text{SHV}}$) and carbapenemases (e.g. *bla*$_{\text{OXA-48}}$, *bla*$_{\text{KPC}}$, *bla*$_{\text{NDM}}$, *bla*$_{\text{VIM}}$).

At present there are at least four commercial real-time PCR tests available for rapid detection of ESBL and/or carbapenemase genes (mostly on rectal swabs). These include: (i) the Check-Direct CPE kit (Check-Points, Netherlands), which targets 4 carbapenemase genes (*bla*$_{\text{KPC-like}}$, *bla*$_{\text{OXA-48-like}}$, *bla*$_{\text{NDM-like}}$, *bla*$_{\text{VIM-like}}$) used together with the Check-MDR ESBL kit (Check-Points, Aageningen, Netherlands) for detection of 3 ESBL genes (*bla*$_{\text{CTX-M-like}}$, *bla*$_{\text{TEM-ESBL like}}$, *bla*$_{\text{SHV-ESBL like}}$), (ii) the Easyplex® SuperBug CPE kit (Amplex Diagnostics GmbH, Mark Gars, Germany) for detection of 5 carbapenemase genes (*bla*$_{\text{KPC}}$, *bla*$_{\text{NDM}}$, *bla*$_{\text{OXA-48}}$, *bla*$_{\text{OXA-181}}$ and *bla*$_{\text{VIM}}$) and 2 ESBL genes (*bla*$_{\text{CTX-M-1}}$ and *bla*$_{\text{CTX-M-9}}$ groups) combined with two extensions (A and B kits) targeting 6 additional *bla*$_{\text{OXA}}$ genes, (iii) the Xpert® Carba-R kit (Cepheid) for detection 5 carbapenemase genes (*bla*$_{\text{KPC}}$, *bla*$_{\text{NDM}}$, *bla*$_{\text{OXA-48}}$, *bla*$_{\text{IMP-1}}$ and *bla*$_{\text{VIM}}$) and (iv) the BD MAX™ CRE assay (Becton Dickenson Diagnostics, MD, Germany) to seek 3 carbapenemase genes (*bla*$_{\text{KPC}}$, *bla*$_{\text{NDM}}$, *bla*$_{\text{OXA-48}}$). These assays can provide results within 45 min-3 hours, informing infection control team. Ther are designed for cultured isolates (the Check-Direct CPE kit) or in the case of the Easyplex® SuperBug CPE kit, the Xpert® Carba-R kit and the BD MAX™ CRE assay for rectal swabs. In principle they could be applied to clinical samples including urine, but have not been validated for this purpose.

Multiplex tandem real-time PCR (MT-PCR)

Multiplex tandem real-time PCR was brought into commercial diagnostics by AusDiagnostics, Sydney, Australia more than 10 years ago. The method combines a real-time PCR in which the progress of reaction can be followed cycle-by-cycle and multiplexing, enabling detection several targets genes in one reaction. The general principle is presented at Figure 13. It is a two-step approach in which multiplexed amplicons of interest are first enriched by PCR. In the second step the products from the multiplex amplification are used as a template for a large number of single-gene PCRs (Stanley & Szewczuk, 2005). The use of two-step amplification minimizes the competition for substrates between individual PCRs in the second step and the formation of primer-dimer products (Stanley & Szewczuk, 2005). Analysis of melting temperatures of PCR products allows specific and easy confirmation that the amplified products obtained are those sought. This method was used to develop a new commercial kit that identities a broad range of antibiotic resistance genes, including not only β-lactamase families but also genes that confer aminoglycoside, trimethoprim and fluoroquinolone resistances in Enterobacteriaceae species within a timeframe of <3 hours. The system incorporates a liquid-handling robot to reduce hands-on-time sample preparation and a real-time PCR amplifier.

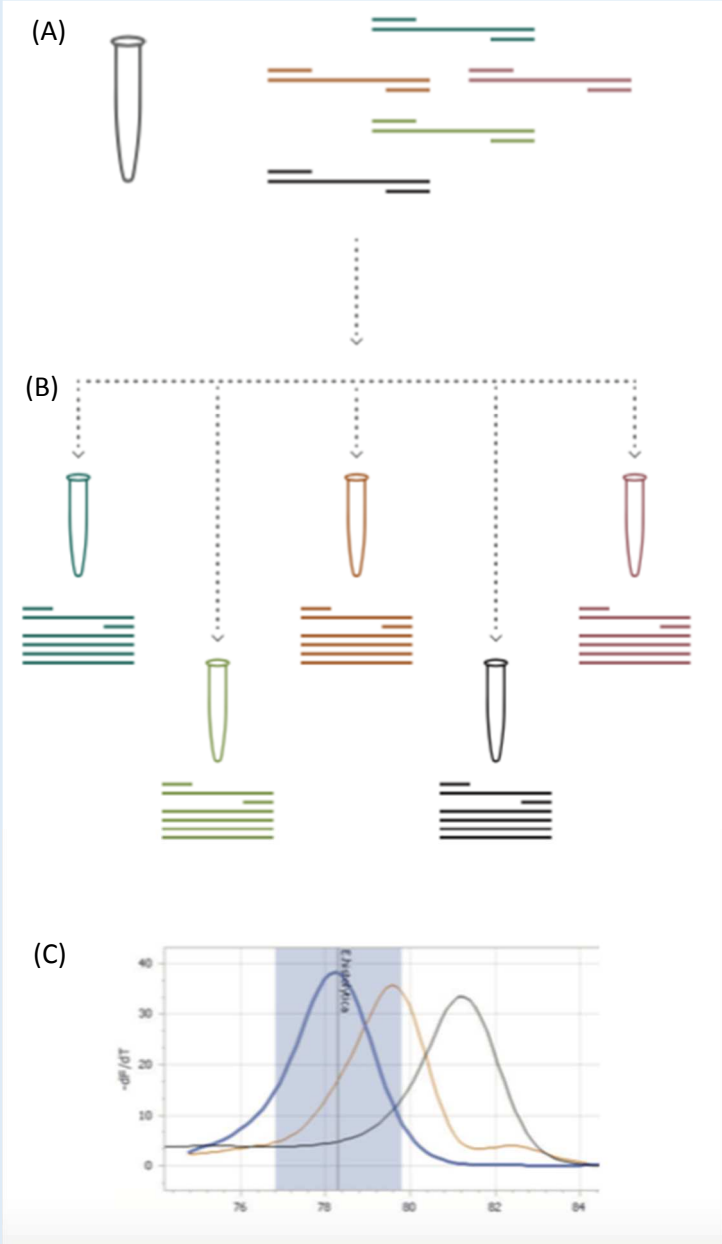


Figure 13. Principle of multiplex tandem PCR.
(A) Amplicons are simultaneously amplified in a multiplexed reaction in a PCR tube for 15-18 cycles;
(B) The PCR products are diluted and divided into a number of real time PCR reactions containing targets and nested PCR primers.
(C) PCR products are analysed based on the melting curves.

1.8.8.2 DNA Microarray technology

Microarray technology offers an alternative to PCR for simultaneous identification of pathogens and their antibiotic resistance genes. The assay contains pre-designed oligonucleotide probes that are bound and immobilized on a solid surface as an array (Lupo *et al.*, 2013). If the targeted allele of a pathogen-specific or resistance gene is present it becomes hybridized to the immobilized probe and generate a fluorescence signal, which is measured and detected by scanner. The technology allows simultaneous detection of a greater number of genes interest compared to PCR-based assays.

Several commercial microarrays have been developed for rapid identification of extended-spectrum β-lactamase (bla_{TEM}, bla_{SHV}, bla_{CTX-M}) and carbapenemase (bla_{NDM}, bla_{VIM}, bla_{KPC}, bla_{OXA-48}, bla_{IMP}) genes in Gram-negative bacteria (e.g. Check-Points ESBL/KPC) and antibiotic resistance gene determinant in *Staphylococcus* spp. (e.g. StaphPlex system Genaco Biomedical Products, Huntsville, USA).

Stuart *et al.* (2010) and Endimiani *et al*. (2010) evaluated microarray platform for ESBL and KPC gene detection. Both studies showed high sensitivity (>95%) and specificity (100%) for the Check-Points ESBL/KPC microarray assays (Cohen Stuart *et al.*, 2010; Endimiani *et al.*, 2010). In another study Batchelor *et al.* (2008) developed a miniaturized microarray for identification of 47 resistance genes in Gram-negative bacteria. These included aminoglycoside, quinolone, trimethoprim, sulphonamide, chloramphenicol, tetracycline and β-lactam resistance genes. The performance of the assay compared well to a PCR method, showing a concordance of 98.8% (Batchelor *et al.*, 2008).

1.8.8.3 Sequencing-based assay

Semi-automated Sanger sequencing has been used in clinical diagnostics for many years. However, the low throughput and high cost of sequencing multiple targets led to the development of new high-throughput next-generation sequencing platforms e.g. Roche 454, Solexa/Illumina, SOLiD and Ion Torrent. The major

advantage of next-generation technology is to yield overall DNA content information on an isolate and to convert it into a comprehensive diagnostic picture. An obvious application for whole genome sequencing (WGS) is epidemiological typing to determine transmission pathways of pathogen and to support outbreak investigation. Salpante *et al.* (2015) explored using the Miseq Illumina platform for strain typing of vancomycin-resistant enterococci (VRE), methicillin-resistant *S. aureus* (MRSA) and multi-drug resistant *Acinetobacter baumannii* and compared results with pulsed-field gel electrophoresis (PFGE). The authors suggest that WGS should replace PFGE as more sensitive method for strain typing (Salipante *et al.*, 2015). In another study Joensen *et al*. (2014) proved that Ion Torrent allowed rapid discrimination between sporadic and outbreak isolates of verocytotoxin-producing *E. coli* (VTEC) (Joensen *et al.*, 2014).

The use of this technology for pathogen profiling and the detection of antibiotic resistance and virulence factor genes has become widespread in reference laboratories. Most sequencing is done using isolated bacteria in pure culture, obviating the challenge of remaining human cells and their DNA. However, Hasman *et al.* (2014) evaluated the Ion Torrent system for sequencing pathogens directly from clinical urines. He found identical resistance genes as in the cultivated pathogens thus removing the 24 h delay needed for pathogen cultivation (Hasman *et al*., 2014).

In another study Bradley *et al*. evaluated the MiSeq Illumina platform to detect antibiotic resistance genes in cultivated isolates of *S. aureus* and *M. tuberculosis* and used these data to predict phenotypic resistance. The authors showed that the error rates for the sequencing method were comparable to “gold standard” phenotypic testing using either automated systems or disc diffusionwith >99% sensitivity and specificity across 12 resistance genes in *S. aureus* and 82.6% sensitivity and 98.5% specificity for 9 antibiotic determinants in *M. tuberculosis* (Bradley et al., 2015).

The Hospital Acquired Infection (HAI) BioDetection system (Pathogenica, Boston MA, USA) is a new commercial NGS-based assay that targets a selection of short DNA regions for rapid identification of the 12 commonest hospital-acquired pathogens and 18 antibiotic resistance genes in a timeframe <12 hour with a detection cut-off $<10^4$ cfu/mL. Veenemans *et al*. (2014) and Arena *et al*. (2014) evaluated this assay for the detection of ESBLs and carbapenemase genes in cultivated isolates. In both

studies the method showed accurate identification of gene presence compared to the reference methods (microarray and multilocus sequence typing, pulsed-field gel electrophoresis, respectively); however the assay could not discriminate ESBL and non-ESBL types of TEM and SHV β-lactamase genes (Arena *et al*., 2014; Veenemans *et al*., 2014).

Ongoing development in sequencing should decrease the cost, accelerate analysis workflow and lead to smaller sequencing devices suitable to busy arranged laboratories. The MinION DNA-based sequencer (Oxford Nanopore Technology) is the exemplar of a new, innovative sequencing technology that will potentially revolutionize the future of diagnostics.

MinION nanopore sequencing

In 2012 Oxford Nanopore Technology, UK released a new platform with real-time analysis of streaming event data on an internet-connected laptop. The major advantages of the approach over other sequencing platform are (i) small size and portability, (ii) relatively low capital cost, (iii) long sequence reads (up to 700 bp, *vs*. 150-300 bp for standard sequencing), facilitating alignment to an existing reference data and assembly of the reads into a genome, providing more reliable information e.g. in plasmid and integron structures and context, (iv) rapid turnaround and real-time data analysis, (v) automation, and (vi) ease-to-use.

The MinION technology is based on the sequencing single-stranded ssDNA as it passes through a protein nanopore placed over an electrical detection grid, as presented on Figure 14. The translocation of the ssDNA through the pores causes a drop in the ionic current that is characteristic of the bases in contact with the pore at that time (Laszlo *et al.*, 2014). The fluctuations in current are sequence dependent and so can be used by a base-calling algorithm to infer the sequence of nucleotides in the DNA (Stoddart *et al.*, 2009; Clarke *et al.*, 2009). This technology is still driven by the developer and it is continually evolving. Preliminary experiments showed that the method can identify microorganism (Kilianski *et al.*, 2015; Wang *et al.*, 2014b), investigate hospital outbreaks (Quick *et al.*, 2014) and predict bacterial antibiotic resistances (Ashton *et al.*, 2015; Bradley *et al.*, 2015; Cao *et al*., 2016; Judge *et al.*,

2015). However, the use of this device directly on clinical samples had not been described prior to the present study. This system potentially provides an alternative approach for pathogen and antibiotic resistance gene identification. In contrast to other PCR-based assays that seek only well known targets it has potential to provide comprehensive genetic data.

(A)

(B)

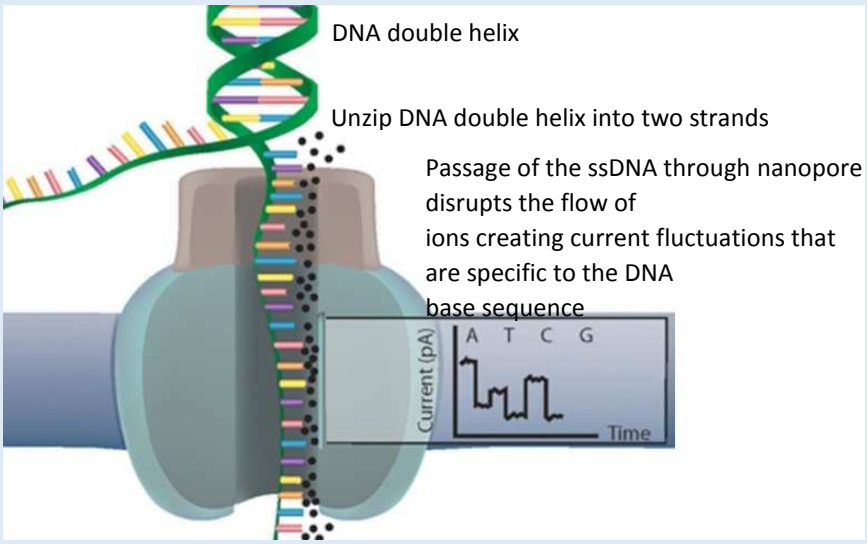


Figure 14. MinION Nanopore sequencer (A) and workflow (B).

1.8.9 Other systems in an early stage evaluation for antimicrobial susceptibility testing

Besides molecular testing there is also interest in developing systems (commercial or otherwise) based on measurement of early bacterial growth curves after exposure to antimicrobial agents. MIC values are thereby assessed in a significantly shorter period compared to conventional antimicrobial susceptibility testing. Bacterial growth can be recorded by digital microscopy, laser scattering technology and biosensor technology. These technologies are in an early stage of evaluation and there is limited published data. Nevertheless they may become valuable diagnostic tools in the future.

Digital microscopy technology

The Accelerate Pheno system (Accelerate Diagnostics, Tuscon, Arizona, USA) is based on cellular analysis of individual bacterial cells and colonies. Microbial cells are captured on a flowcell channel surface using a mild electrical charge. In situ hybridization with target probes is utilized for bacterial identification. MICs are determinated by analyzing of growth/lysis/morphology of individual cells to a single antibiotic concentration, with results available within 7 h. Price *at al.* (2014) evaluated the performance of this automated microscopy system for immobilized *S. aureus* cells, seeking to detect the MRSA phenotype and clindamycin resistance against a conventional disk diffusion method. They showed that the system could perfectly discriminate MRSA and MSSA phenotypes and, in the majority of cases also detect clindamycin resistant isolates (Price *et al.*, 2014).

Laser scattering technology

There are two commercial systems based on laser light scattering technology that allow antibiotic susceptibility testing directly on urine specimens, though they cannot identify bacteria. These are the Alfred60 (Alifax, Italy) and BacterioScan™ 216Dx (Kibbutz Eilon, Western Galilee, Israel) systems. Both monitor the intensity of bacterial replication, providing real-time bacterial growth curves. The Alfred60

system uses specific culture broth bottles (Uro-Quick detection kit) whilst the BacterioScan™ 216Dx utilizes specific microcuvettes. Roveta *et al*. (2004) evaluated the Uro-Quick system for antimicrobial resistance profiling for the main agents used to treat UTI directly on urine samples. The study showed good agreement (>90%) with a disc diffusion reference method. Results were available within 5 h (Roveta *et al.*, 2004). In another study Zerda *et al*. (2015) compared the BacterioScan system against conventional phenotypic method with EUCAST breakpoints again directly on urine specimens. The system showed 92% sensitivity and 81% specificity for urine with bacterial counts >10^4 cfu/mL and data were available within 3 h (De la Zerda *et al*., 2015).

Biosensor systems

A molecular biosensor device has two components: a recognition element (enzyme, antibody or DNA) and a transducer. The target specimen is bound to the recognition element, generating a measurable signal (light or an electrical current) which is detectable via the transducer (CCD camera, photodiode, electrode). The in-house UTI Sensor Array is an example of a biosensor for uropathogen profiling with a cut-off of 10^4 cfu/mL, whilst the biosensor-based AST was designed for susceptibility testing (Mach *et al.*, 2011). In this latter assay, 16S rRNA probes are utilized to monitor bacterial growth and to allow identification and antimicrobial testing. Species-specific DNA probes targeting the most common uropathogens are immobilized on the sensor surface and are used to measure growth of the pathogen under different antibiotic conditions. Biosensor signals from samples incubated with an antibiotic are compared to those from samples incubated without antibiotic.

Cell Phone-based microphotometric systems

This technique compromises three major components i) gas-permeable microwell arrays with antibiotics precoated on the wells, ii) a colorimetric indicator of cell viability to monitor bacterial growth and iii) a cell phone–based microphotometric system.

The system was developed to rapidly detect bacterial growth and allows antimicrobial susceptibility testing in a microwell array directly from urine samples with bacterial concentrations of 10^1-10^6 cfu/mL (Kadlec *et al*., 2014). The assay was designed for point-of-care applications in a low-resource settings as it does not required complicated sample procedures. Previously Johnson *et al.* (1985) showed the utility of a colorimetric indicator to determinate the antibiotic susceptibilities of Gram-negative bacteria. The authors used tetrazolinum (triphenyltetrazolium chloride), an organic salt dye reduction, as a colorimetric indicator of bacterial growth that converts to a red colour in the presence of reducing substances (Johnson *et al.*, 1985).

Microfluidic system

These small called 'lab-on-a-chip' platforms (LOC) are portable and utilize small volumes of reagent and test analyte. They might be suitable for rapid point-of-care detection of antibiotic susceptibility. Microfluidic systems are in an early phase development but so far have been shown able to assess MICs using (i) microfluid agarose channels (Chen *et al.,* 2010), (ii) electrochemical quantification of 16S rRNA, without the need to perform amplification steps (Riahi *et al.,* 2011) or (iii) a microfluidic pH sensor (Pulido *et al*., 2013). In all those cases susceptibility testing results were available in 3-4 h.

Rapid EUCAST disc diffusion test

Recently it has been showed that disc diffusion test can reliably be read after 6 h of incubation. Fröding *et al*. (2017) evaluated phenotypic tests for production of ESBLs and carbapenemases using tablet kits from ROSCO Diagnostica A/S (Taastrup, Denmark) and Mast Group Ltd (Bootle, UK) on multi-drug resistant Enterobacteriacea based on the EUCAST methodology. The study demonstrated that inhibition zones for extended-spectrum cephalosporins could be interpreted after 6 h. The authors showed that more than 80% of ESBL and non-ESBL producing Enterobacteriacea gave reliable results for ceftazidime, cefotaxime, ciprofloxacin, gentamicin and meropenem, but

carbapenem-producing strains needed a full 18-20 h of incubation (Fröding *et al.*, 2017).

1.9 Rationale and aims of the research

1.9.1 Rationale

Rapid identification of *E. coli* and other common uropathogens, together with antibiotic resistance profiling are desirable in both community and hospital settings in order to: (i) decrease the number of hospital admissions for complicated UTIs, (ii) minimize the risk of treatment failure or size effects (e.g. *C. difficile*) caused by inadequate or excessive empirical therapy, (iii) decrease the further spread of strains resistant to standard antibiotics used in the treatment of uncomplicated UTIs, (iv) conserve the antibiotics presently reserved for treatment of difficult nosocomial infections and (v) shorten patients' hospitalization.

Conventional testing needs cultivated bacteria, meaning c. 24-72 h delay before targeted therapy can be deployed. Review of the current literature identified several potential rapid systems for faster pathogen identification and susceptibility testing through some are limited to only detecting well-known targets. What is desirable, at least for severe infection leading to hospitalization, is to develop comprehensive methods enabling fast pathogen identification and resistance profiling directly from urine, without culture. To achieve this two approaches were evaluated:

Firstly, MALDI-TOF (Matrix-Assisted Laser Desorption Ionization Time-of-Flight) mass spectrometry for direct bacterial identification from urine together with multiplex tandem PCR for resistance gene profiling. MALDI-TOF was also evaluated for rapid β-lactamase detection and profiling directly from urines and their cultivated isolates

Secondly, MinION sequencing for simultaneous bacterial and resistance gene identification, again directly from urine.

As background to developing these rapid methodologies, a surveillance of uropathogens from the Norfolk and Norwich University Hospital in July and

November 2014 was performed to investigate the local aetiology, epidemiology, and antimicrobial resistance of UTIs.

Overall the studies aimed to develop two approaches that might significantly shorten the time needed for UTI diagnostics to <5 hours, and thereby to enable earlier appropriate targeted antimicrobial therapy, within the typical dosage interval of a q8h therapy.

1.9.2 Aims of the research

- To determinate the local aetiology, epidemiology and antibiotic resistances of UTIs at the NNUH and in the community.
- To indicate patient groups and locations where a rapid diagnostic could be implemented.
- To analyze seasonal variations of UTIs.
- To develop a MALDI-TOF mass spectrometry method for rapid identification of pathogens directly from clinical urines, without culture. For this purpose, different sample preparation procedures to deplete human cells were evaluated.
- To develop a MALDI-TOF assay for detection of β-lactamase (ESBL/AmpC) activity directly from urine specimens and their cultivated isolates.
- To develop and evaluate multiplex tandem PCR assays for rapid detection of the commonest antibiotic resistance genes in Enterobacteriaceae directly from urine specimens, without culture.
- To develop a nanopore sequencing technology using the MinION device for rapid simultaneous detection of uropathogens and their antibiotic resistance genes directly from clinical urines. To allow this, also a sample preparation procedure to enrich bacterial DNA was investigated.
- To propose possible fast diagnostic workflows to detect trimethoprim-resistant *E. coli* in uncomplicated UTIs in the primary care.
- To propose possible rapid diagnostic workflows for identification of *E. coli* and other common uropathogens together with their antibiotic resistance profilies.

Chapter 2

MATERIALS AND METHODS

2.1 Epidemiology of UTI specimens at the NNUH

An epidemiological study was performed based on urines processed in the clinical microbiology laboratory of the Norfolk and Norwich University Hospital in the months of July and November 2014. All urine specimens (July n= 9558; November n= 8991) including those triaged out on account of low bacterial counts $<10^5$ cfu/mL were analysed.

Submitted urines undergo a screening process on an automated iQ®200 (Iris Diagnostics, Chatsworth, CA, USA) analyser, which captures and reviews flow cell digital images using automatic particle-recognition software. Particles are classified on the basis of texture, contrast, shape and size (e.g. as WBC, RBC or bacteria). Figure 15 shows the urine processing performed in the NNUH.

Those urines meeting the criteria for culture are plated on BD CHROM agar Orientation Medium (Becton Dickinson, Oxford, UK) and incubated at 37^{o}C overnight. Species or bacterial groups were identified according to the colouration of growth on this medium which is described in Section 1.8.6 (Classical bacterial identification-Chromogenic media).

In vitro susceptibility testing was primarily performed by the BSAC disc diffusion method using commercially discs from Oxoid, (Basingstoke, UK) (see Table 4).

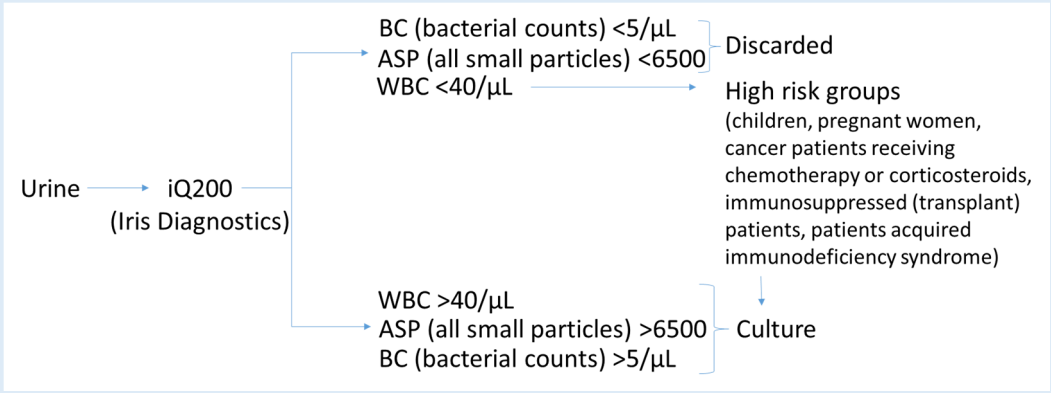


Figure 15.Urine processing performed at the NNUH.

Table 4. Antibiotic susceptibility discs used for susceptibility testing.

Antibiotic susceptibility discs	**Disc content**
First line antibiotics	
Amoxicillin	10 µg
Co-amoxiclav	20 µg + 10 µg
Cefpodoxime	10 µg
Cephalexin	30 µg
Gentamicin	10 µg
Nitrofurantoin	200 µg
Piperacillin/Tazobactam	75 µg + 10 µg
Trimethoprim	2.5 µg
Second line antibiotics	
Fosfomycin	50 µg
Ertapenem	10 µg
Meropenem	10 µg
Ciprofloxacin	1 µg
Ceftazidime	30 µg
Cefuroxime	30 µg

2.1.1 Statistics

Epidemiological data were analysed using two statistical tests of significance. These were (i) significance of the changes in population proportions and (ii) chi-square test to compare observed data with expected data. Both tests were applied to determin whether there was significant variation between July and November in (i) the total number of urines submitted to the microbiology laboratory, (ii) the numbers of urines submitted by locations and the results from investigations, (iii) the distribution of the Gram-positive *vs.* Gram-negative isolates by the patients' age and gender and (iv) the resistance rates for amoxicillin and trimethoprim for different locations.

The test of significance for differences in population proportions was performed in two steps. Firstly, the null (H_0) and alternative (H_a) hypotheses were assessed based on following assumptions:

(i) The null hypothesis H_0, states that there is no difference in the population proportions between two investigated groups

$$H_0 : p_1 - p_2 = D_0$$

where $D_0=0$

(ii) The alternative hypothesis H_a contains the reliable values of the parameter if the null hypothesis is rejected. H_a may be written as one of the following 3 possibilities:

$$H_a : p_1 - p_2 < D_0 \text{ (Lower-tail test)}$$

$$H_a : p_1 - p_2 > D_0 \text{ (Upper-tail test)}$$

$$H_a : p_1 - p_2 \neq D_0 \text{ (Two tailed test)}$$

Secondly, the test statistic was calculated using the formula:

$$z = \frac{(\hat{p}_1 - \hat{p}_2) - D_0}{\sqrt{\tilde{p}(1-\tilde{p})\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}}$$

$$\tilde{p} = \frac{x_1 + x_2}{n_1 + n_2}$$

where:

x_1, x_2- the number of submitted urines in a specified location or for a given group in July and November respectively;

n_1, n_2- the total number of submitted urines in July and November respectively;

$\hat{p}_1, \hat{p}_2$- relative proportions (%) of submitted urines in a specified location or for a given group in July and November, respectively.

The test statistic *z* is normally distributed if the null hypothesis H_0 is true. The *p*- value associated with *z* was determinated based on the formula above. If the *p* was less than the significance level (ordinarily p <0.05) the null hypothesis was rejected and the difference assigned to be statistically significant or if the *p*- value was higher than the significance level (ordinarily p >0.05), we cannot reject the null hypothesis and the differences in the population are not statistically significant.

The second statistical approach utilized the chi-square (X^2 or chi^2) test to determine if there was a significant relationship between two nominal variables. The distribution of one nominal variable, given different values of the second nominal variable, were compared and test aimed to verify whether the differences in the conditional distributions was significant. The procedure of the test includes the following steps:

Firstly, the chi-squared test statistic resembling a normalized sum of squared deviations between observed and predicted frequencies was calculated using the formula:

$$\chi^2 = \sum_{i=1}^{n} \frac{(O_i - E_i)^2}{E_i} = N \sum_{i=1}^{n} \frac{(O_i/N - p_i)^2}{p_i}$$

where:

Oi- the number of observations e.g. the total number of urines submitted for GP in July or November;

N- the total number of observations;

Ei- the expected (theoretical) frequency of type *i*, asserted by the null hypothesis that the fraction of type *i* in the population is *pi;*

n- the number of cells in the table.

The number of the result of the test was selected compared to the critical value from the chi-squared distribution for the appropriate number of degrees (ordinarily one fewer than the number of categories being compared) of freedom and the selected confidence level. The null hypothesis (H_0) of independence was rejected when the test statistic was higher than the critical value corresponding to the assumed significance level.

2.2 Matrix-Assisted Laser-Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) to identify bacteria from urine specimens

All MALDI-TOF analysis was performed on a Microflex LT instrument (Brüker Daltonics GmbH, Leipzig, Germany) with FlexControl 3.4 software (Brüker Daltonik GmbH) for visual inspection of the mass spectra. The spectra were recorded in the linear positive mode at a laser frequency of 60 Hz within a mass range from 2-20 kDa**.** The trigger delay time was 10 µs. For each spectrum**,** 800 laser shots were collected and analyzed (4×200 laser shots from different positions of the target spot). Automated analysis of the raw data, involving database searching and bacterial identification, was performed by MALDI Biotyper Real Time Classification software and MALDI Biotyper OC 3.1 (Brüker Daltonik GmbH). The results were analyzed across a mass range from 4-15 kDa giving 'score values' between 0 and 3, reflecting the similarity between the sample and the reference spectrum. The display gives the top 10 matching database records. Ordinarily a score of <1.699 generally indicates no reliable identification, a score 1.700-1.999 indicates genus level, and >2.000 gives species identification. We reviewed the use of lower criteria to potentially increase the number of direct identifications from urine. In particular we took a score value <1.450 to indicate no identification; a score of 1.450-1.600 to indicate genus level, and >1.600 for species identification.

All identification were performed in duplicate. *E. coli* (BTS- bacterial testing standard, Brüker Daltonics GmbH) was used as a standard to calibrate the instrument for all experiments. Calibration points with a reference mass compromised RL29 [M+2H]2+ (3637.8 Da), RS32 [M+H]+ (5096.8 Da), RS34 [M+H]+ (5381.4 Da), RS33meth [M+H]+ (6255.4 Da), RL29 [M+H]+ (7274.5 Da), RS19 [M+H]+ (10,300.1 Da), RNAse A [M+H]+ (13,683.2 Da), Myoglobin [M+H]+ (16,952.3 Da). As a starting point for direct tests on urines we used the method of (Ferreira *et al.*, 2010) described in Figure 16 and sought to improve it.

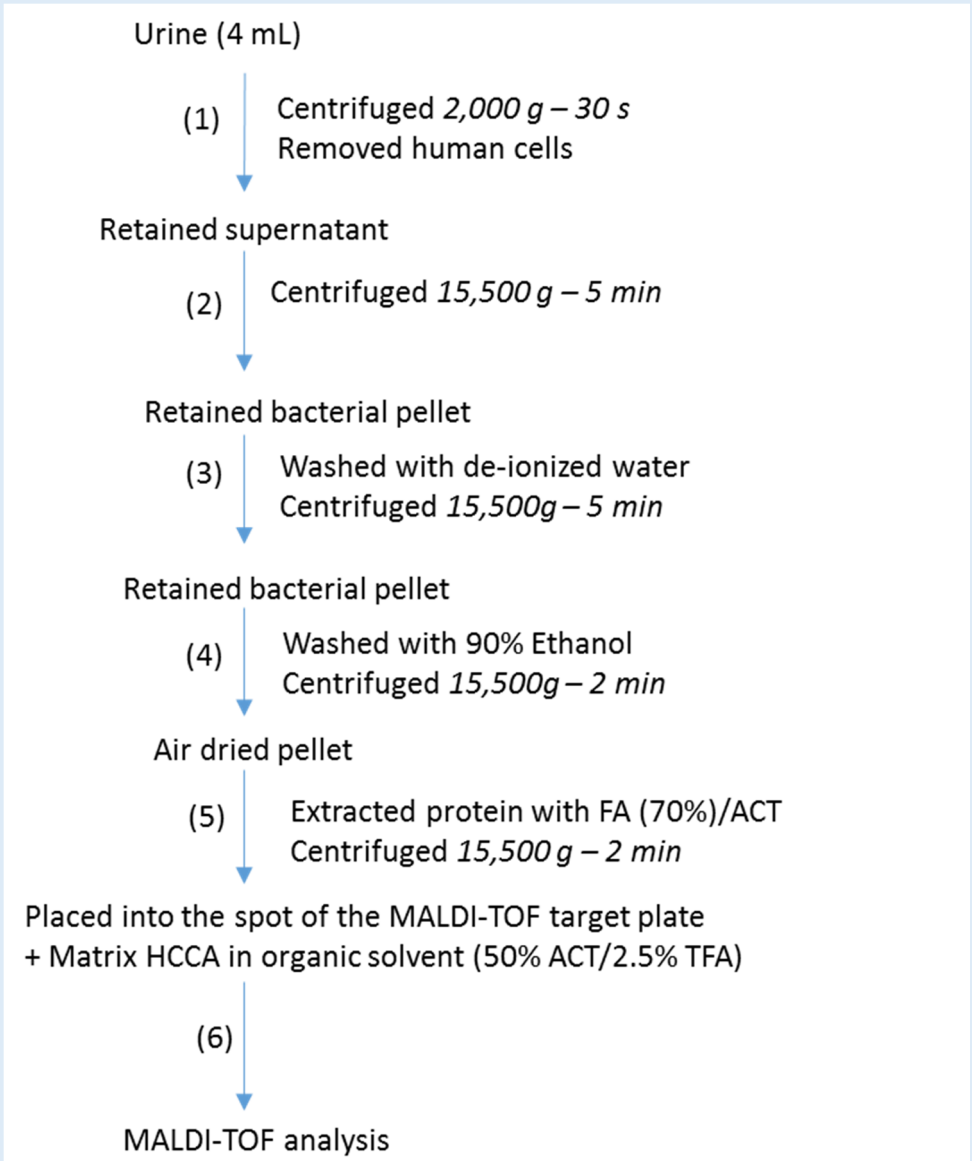


Figure 16. Initial sample preparation protocol for MALDI-TOF identification of bacteria from urine by (Ferreira *et al.*, 2010).

For abbreviations see page number xvi.

2.2.1 Optimisation of the sample preparation procedure for pathogen identification for MALDI-TOF

In order to optimise the MALDI-TOF procedure for rapid identification of pathogens directly from urine samples (see Figure 16), several factors were varied (Table 5).

Table 5. Optimization of parameters for rapid bacterial identification directly from urine specimens (steps refer to Figure 16).

Factors	Variations
Volumes of urine specimen	2 mL, 5 mL, 10 mL
Centrifugation period (step 1)	30 s, 2 min, 5 min
Centrifugation speed (step 1)	2000 rpm (300 *g*), 3000 rpm (600 *g*), 4000 rpm (1100 *g*), 5000 rpm (2400 *g*)
Ethanol volume (step 4)	300 µL, 600 µL, 900 µL
Chemical reagents (step 2)	Addition of Anionic Acid Labile Surfactant II (AALS II) (Protea Biosciences, Morgantown, WV, USA), lysis buffer: Pierce IP (immunoprecipitation), and CM (mammalian cells), 0.5% and 1% SDS (sodium dodecyl sulphate), lysozyme (1 mg/mL)
Extraction reagents (step 5)	B-PER extraction buffer (Thermo fisher), 70% formic acid/acetonitrile, formic acid/2-propanol, acetonitrile/trifluoroacetic acid (1:1), acetonitrile/trifluoroacetic acid (2:1)
Commercial kit (variously at step 1-5 following manufacturer's instruction)	Sepsityper kit (Brüker Daltonik GmbH)

Further parameters considered included (i) the sensitivity of the instrument, (ii) the effect of boric acid (1-2% used as a transport preservative) in urine, (iii) the effect of antibiotics (ciprofloxacin, trimethoprim and trimethoprim/sulfamethoxazole (co-trimoxazole)) in the urine, and (iv) the different ratios of two bacterial strains present in the urine. In each experiment single colonies from the plates containing cultivated Gram-negative or Gram-positive bacteria (*E. coli, K. pneumoniae, P. mirabilis, P. aeruginosa*, *E. faecalis,* and *S. aureus*) were inoculated in LB (Luria-Bertani, Sigma, St. Louis, USA) broth for overnight at 37^{o}C. One millilitre volumes of the overnight cultures at a density between 10^{8} - 10^{9} cfu/mL were then spiked into 9 mL of uninfected urine from a healthy volunteer. Two millilitres (or other volumes see Table 5) of the diluted 'spiked urine' were used for MALDI-TOF analyses according to the protocol shown in Figure 16.

Detection limit

To assess the minimal bacterial concentration that allowed reliable MALDI-TOF identification, serial dilutions from 10^{-1} to 10^{-4} of overnight LB cultures were performed in urine and analysed as shown in Figure 16.

Effect of boric acid

The effect of boric acid on MALDI-TOF scores was investigated because most clinical urine specimens are collected in containers with 1-2% boric acid, as a sample preservative, added to prevent bacterial overgrowth during transport to the laboratory, and allowing satisfactory culture and microscopy when analysis is performed up to 48 h later. To determine the effect of boric acid on MALDI-TOF scores urines from boric acid- containing and non-containing tubes were analyzed for up to 7 days storage in the fridge (4-8°C), using the extraction procedure with FA (70%)/ACT presented in Figure 16 (step 5), and without extraction (i.e direct spotting of the washed bacterial pellet >10^{5} cfu/mL onto the MALDI-TOF target, after step 3, Figure 16).

Effect of antibiotics

The presence of antibiotics in urine might affect MALDI-TOF analysis. To investigate this possibility ciprofloxacin, trimethoprim and trimethoprim/sulfamethoxazole (1:5) (co-trimoxazole) at concentrations of 100 mg/L, 200 mg/L, 600 mg/L (100 mg/L + 500 mg/L), respectively, were added to urine spiked with bacteria (10^9 cfu/mL) before repeated analysis on MALDI-TOF for 6 days storage in the fridge (4-8°C).

Presence of multiple organisms

To ascertain ranges where MALDI-TOF detected one or both pathogens present in a mixture of overnight broth cultures, urine was spiked with two organisms in the ratios (volume): 1:1; 1:2; 1:3; 1:4; 1:10; 2:1; 3:1; 4:1; 10:1 using bacterial suspension with densities: 0.26 x 10^9 cfu/mL; 0.33 x 10^9 cfu/mL; 0.5 x 10^8 cfu/mL; 0.15 x 10^9 cfu/mL for *E. coli*; *P. aeruginosa*; *E. faecalis* and *S. aureus* respectively. These samples were then analysed on the MALDI-TOF as shown in Figure 16.

Effect of bacteria-lysing agents

In an attempt to improve MALDI-TOF analysis, SDS (sodium dodecyl sulphate) and the enzyme lysozyme (only with Gram-positive bacteria) were added. Two millilitres of clinical urine sample with high concentrations of white blood cells (>10^9 WBC/L) were processed followed the extraction procedure (Figure 16) but with the addition of 1% SDS (50 µL) and 15 µL of lysozyme (1 mg/mL) to the bacterial pellet obtained (step 2). This was then immediately re-centrifuged (step 3), except for Gram-positive isolates where the mixture was first held for 30 min at 37^{O}C with lysozyme.

2.2.2 Rapid identification of pathogens directly from clinical urines by MALDI-TOF

A pilot study was undertaken with clinical urine samples from the Microbiology Department of NNUH to evaluate the use of MALDI-TOF for the clinical laboratory investigation of urinary infection. Urines included in the study were selected based on culture results or Iris screening, aiming to investigate the detection of various Gram-positive and Gram-negative bacteria, whereas consecutive sample were dominated by *E. coli*.

One hundred fifty clinical urine samples were analysed. These were from in- and out-patients and standardly were collected into boric acid containers for routine clinical laboratory analysis. Each sample first underwent routine screening using the automated iQ®200 (Iris Diagnostics) analyser, with culture then performed, (or not) based on the results according to standard laboratory protocols presented in Figure 15.

Selected samples (96 culture-positive; 27 culture-negative but microscopy positive, and 27 heavy mixed bacterial growth) were run on the MALDI-TOF using the optimised method (see Figure 17). Samples that gave discrepant identifications between the clinical laboratory results and the direct MALDI-TOF analysis were re-cultured and re-identification was performed from single colonies by MALDI-TOF.

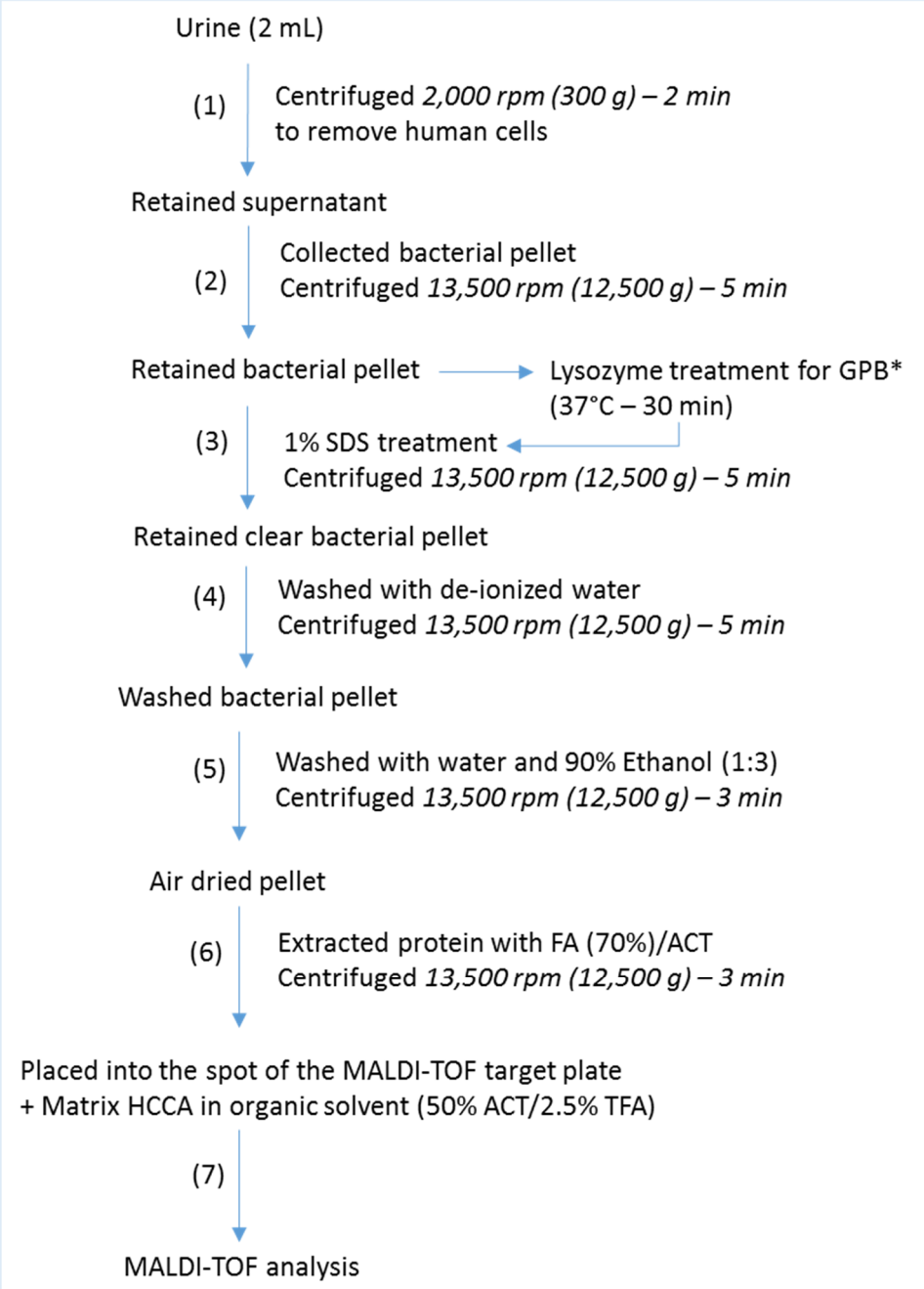


Figure 17. Optimised procedure for direct bacterial identification on MALDI-TOF.

Identification was performed by clinical laboratory based on the culture plated on the BD Orientation™ agar.

*GPB- Gram-positive bacteria

2.2.3 Rapid discrimination of β-lactamase-producing pathogens directly from urine and cultivated isolates by MALDI-TOF

2.2.3.1 Optimization of assays of cephalosporins hydrolysing activity

In order to optimize methodology for the detection of cephalosporin hydrolysis activity directly from urine and from cultivated isolates on the MALDI-TOF, two parameters- incubation time and antibiotic diluent buffers-were varied.

Antibiotic diluent buffer

We assessed three buffers as dilutions for cephalosporins, namely (i) 10 mM ammonium phosphate pH 7.2, (Sigma-Aldrich, St. Louis, USA), (ii) 10 mM ammonium bicarbonate pH 8-9 (Sigma-Aldrich), and (iii) 10 mM ammonium hydrogen citrate pH 7.2 (Sigma-Aldrich). Ten-fold concentrated solution of ceftazidime (Sigma-Aldrich, 0.25 mg/mL) and cefotaxime (Sigma-Aldrich, 0.5 mg/mL) were diluted in these buffers. Then 50 µL of antibiotics solution was added to sufficient cultivated bacteria to fill a 1 µL inoculation loop. Multi-resistant *E. coli* H141480453 with NDM-4 and OXA-181, and penicillin- and cephalosporin-susceptible *E. coli* recovered from a clinical urine were used as positive and negative controls. These mixtures were incubated at 37^{o}C with shaking at 900 rpm for 2 hours (cefotaxime), and 4 hours (ceftazidime). Subsequently, the mixtures were centrifuged at 13,500 rpm for 3 minutes to pellet the bacteria and 1 µL of the supernatant was placed on the MALDI-TOF target plate as two replicates, dried at room temperature, and overlaid with MALDI matrix (10 mg/mL of cyano-4- hydroxy-cinnamic acid [HCCA] in 50% acetonitrile – 0.1% trifluoroacetic acid (Brüker Daltonik, Bremen). MALDI-TOF analysis was performed as described in Section 2.2.3.2 (MALDI-TOF MS analysis of the hydrolysis assays).

Incubation time

To assess the incubation time required for cephalosporin hydrolysis assays cultivated isolates of β-lactamase positive and negative controls filling a 1 µL inoculation loop were mixed with following antibiotics: cefpodoxime (Sigma-Aldrich, 0.25 mg/mL), cefepime (Alfa Aesar, Ward Hill, USA; 0.5 mg/mL), ceftriaxone (Alfa Aesar, 0.5 mg/mL), cefotaxime (0.5 mg/mL), and ceftazidime (0.25 mg/mL) diluted in 50 µL of 10 mM ammonium citrate buffer pH 7.2 (Sigma Aldrich). These suspension, and the same antibiotics without bacteria, were incubated at 37°C with shaking at 900 rpm for different time periodd: 0.5 h, 1 h, 1h 30 min, 2 h, 2h 30 min, 3h, 3h 30 min, 4h.

Multi-resistant *E. coli* H141480453 harbouring β-lactamase genes (bla_{NDM-4} and $bla_{OXA-181}$) and *E. coli* strain J53 producing TEM-10 (only with ceftazidime assay) were used as positive controls. *E. coli* recovered from clinical urine and fully susceptible to penicillins and cephalosporins was used as a negative control in each of the assays.

After appropriate incubation the samples were centrifuged at 13,500 rpm for 3 min. The supernatant was then subjected to MALDI-TOF analysis as in Section 2.2.3.2 (MALDI-TOF MS analysis of the hydrolysis assays).

2.2.3.2 MALDI-TOF MS analysis of the hydrolysis assays

MALDI-TOF analysis was performed on a Microflex LT instrument (Brüker Daltonics GmbH). Firstly, with FlexControl 3.4 software and secondarily with MALDI Biotyper Compass version 4.1.40 (MBT-STAR-BL) software (Bruker Daltonik GmbH). The former software was used for all antibiotics, the latter only for ceftazidime and cefotaxime. The spectra were recorded in the linear positive mode at a nitrogen laser frequency of 60 Hz, with a low mass range from 100 Da to 1,000 Da. For each spectrum**,** 240 laser shots were collected and analyzed (6×40 laser shots from different positions of the target spot). An 'antibiotic standard' MBT STAR-ACS (Bruker Daltonik GmbH) consisting of reference spectra of bradykinin (1-7) [M+H]+ (757.39 Da), bradykinin (1-5) [M+H]+ (573.31 Da), Lys-Lys-Lys [M+H]+ (403.30 Da), and Ser-His [M+H] (243.10 Da) was used for automated calibration.

2.2.3.3 Data analysis

FlexAnalysis version 3.4 software (Bruker Daltonik GmbH) was used for manual analysis of the peak profiles for all antibiotics. Spectra were smoothed (SavitzkyGolay; width, 0.2 *m/z*; cycles 1 algorithm) and the baseline subtracted (TopHat algorithm). Only peaks corresponding to the antibiotic were labelled.

Automated analysis was performed using commercial Bruker's MBL STAR-BL software for ceftazidime and cefotaxime only, and the prototype MBT STAR-BL hosted on http://mbtprot.bdal.de/MSBL for all antybiotics tested.

Review of the peak patterns for the native and hydrolysed forms of the different cephalosporins led to characteristic profiles for β-lactamase producing and non-producing strains. Changes in the peak intensity ratios within a spectrum allowed quantitative evaluation of hydrolysis measured as logarithm RQ (resistance quotient; rate of hydrolysis).

Isolates were classified as negative for cephalosporin hydrolysing β-lactamases and potentialy susceptible if the peak intensity distributions corresponding to the native (non-hydrolysed) cephalosporins and were similar to those for the negative control. Isolates with cephalosporin-hydrolysing β-lactamases were classified as potentialy resistant if the intensities of peaks corresponding to the hydrolysed cephalosporin were similar to those for the positive control. Isolates showing an intensity distribution between the negative and positive controls were called 'slow hydrolyses'.

2.2.3.4 Detection of cephalosporin-hydrolysing activity directly from urine and cultivated bacteria

Assays were performed with final concentration of the following cephalosporins: ceftazidime (0.25 mg/mL), cefotaxime (0.5 mg/mL), cefpodoxime (0.25 mg/mL), cefepime (0.5 mg/mL), and ceftriaxone (0.5 mg/mL). The concentrated stocks were dissolved in 10 mM ammonium hydrogen citrate, pH 7.5 (Sigma-Aldrich).

Assays (Section 2.2.3.5) were performed with 91 infected clinical urines from the Microbiology Department of the NNUH, and on the isolates recovered from these specimens. Samples were selected based on phenotypic testing results in order to

investigate the detection of β-lactamase producing Enterobacteriaceae particularly those with extended-spectrum β-lactamase enzymes (ESBLs) and AmpC β-lactamases.

Forty-three urines of the total 91 samples were selected as Enterobacteriaceae producing extended-spectrum β-lactamase enzymes (ESBLs) and twenty-two were AmpC β-lactamase producers based on the NNUH laboratory disc results showing synergy between cefpodoxime with clavulanate as a ESBL inhibitor and/or cloxacillin as a AmpC inhibitor. Twenty-six isolates were full susceptible to 3rd generation cephalosporins. Multi-drug resistant *E. coli* strain H141480453, which produced TEM-1, NDM4, OXA-181, CTX-M-15 and CMY-2 β-lactamases was used as a positive control for assays with cefotaxime, cefepime, ceftriaxone, and cefpodoxime, and *E. coli* strain J53 with TEM-10 enzyme as a positive control with ceftazidime. The AmpC derepressed strain *Enterobacter cloacae* 684 (Yang *et al.*, 1988) was used as an AmpC control. *E. coli* recovered from clinical urine and fully susceptible to penicillins, and cephalosporins was used as a negative control, tested in parallel.

The presence of β-lactamase genes was confirmed by Illumina genomic sequencing for 51 of the 91 specimens including all the isolates with ESBL phenotypes; penicillins and cephalosporins resistances were sought by phenotypic profile for all samples, as in Section 2.2.3.6.

2.2.3.5 Sample preparation for MALDI-TOF hydrolysis assay

Clinical urines (1-1.5 mL) were centrifuged at 300 *g* for 2 min to deplete human cells. The supernatant was collected, re-centrifuged at 12,300 *g* for 5 min, and the resulting bacterial pellet washed in 300 µL of molecular grade water, and then re-centrifuged at 12,300 *g* for 3 min. Sufficiant washed bacterial pellet from urines or fresh overnight bacterial isolate growth on the agar to fill a 1 µL inoculation loop was re-suspended in 50 µL of the antibiotic solution and incubated at 37^{o}C shaking at 900 rpm for 2 hours (except ceftazidime, 4 hours). Subsequently, the samples were centrifuged at 12,300 *g* for 3 min, and then two 1 µL replicate volumes were spotted onto the polished steel MALDI-TOF target plate, dried at room temperature and

overlaid with MALDI matrix (10 mg/ml of cyano-4- hydroxy-cinnamic acid [HCCA] in 50% acetonitrile – 0.1% trifluoroacetic acid (Brüker Daltonik).

Positive and negative controls and antibiotic solution without bacteria were run in parallel with used for each assays. Data analysis was performed as described in Section 2.2.3.2 and 2.2.3.3.

2.2.3.6 Phenotypic characterization of uropathogens

Bacteria from the clinical urines were cultivated on the BD CHROM agar Orientation Medium and CLED agar plates (Becton Dickinson), incubated at 37^{o}C overnight. Species identification was performed based on the colouration of growth (see Section 1.8.6) and MALDI-TOF mass spectroscopy (see Section 2.2).

In vitro susceptibility testing was primarily performed by the BSAC disc diffusion method using commercial discs from Oxoid (Table 6). Extended-spectrum β-lactamase and AmpC phenotypes were detected using the Mastdiscs^{TM} D68C AmpC & ESBL Detection Set (Mast Group, Bootle, UK) and combination disc diffusion test (CDT) with an additional cefoxitin (FOX) disc. Mast's ESBL and AmpC Set (D68C) contains four discs: (A) cefpodoxime 10 µg, (B) cefpodoxime 10 µg + ESBL inhibitor (clavulanate), (C) cefpodoxime 10 µg + AmpC inhibitor (cloxacillin) and (D) cefpodoxime 10 µg + both ESBL and AmpC inhibitors. Interpretation was performed according to the manufacturer's instructions. Further investigations utilized the Mastdiscs D63C Set (Mast Group) which includes cefepime (30 µg) and cefepime with clavulanic acid (30 µg + 10 µg) for ESBL confirmation in isolates with chromosomal AmpC, and D69C Set (Mast Group) which includes three discs: (A) cefpodoxime 10 µg + AmpC inducer, (B) cefpodoxime 10 µg + AmpC inducer + ESBL inhibitor, and (C) cefpodoxime 10 µg + AmpC inducer + ESBL inhibitor + AmpC inhibitor for detection plasmid-mediated and chromosomal AmpC, whether inducible or derepressed.

All susceptibility test were done using fresh overnight cultures of isolates recovered from clinical urines, with controls.

Table 6. Antibiotic susceptibility discs used for susceptibility testing.

Antibiotic susceptibility discs	Disc content
Amoxicillin	30 µg
Co-amoxiclav	20 µg + 10 µg
Cefpodoxime	10 µg
Cephalexin	30 µg
Ceftazidime	30 µg
Cefuroxime	30 µg
Cefotaxime	30 µg
Cefepime	30 µg
Cefoxitin	30 µg
Piperacillin/tazobactam	75 µg + 10 µg

2.2.3.7 Library preparation and Illumina genomic sequencing of ESBL-and AmpC-producing isolates cultivated from urines

Fifty-one of the cultivated isolates (41 containing putative ESBL producers and 10 putative AmpC producers) recovered from clinical urines were genomically sequenced on the MiSeq system (Illumina, Cambridge, United Kingdom). Single colonies from CLED plates (Oxoid) were inoculated in 10 mL amount of LB broth (Sigma-Aldrich) at 37^{o}C overnight. Then 500 µL of culture at a density between 10^8 - 10^9 cfu/mL, were centrifuged at 12 300 *g* for 5 minutes. The resulting bacterial pellet was re-suspended in 200 µL of lysis buffer (Roche), 25 µL proteinase K (20 mg/mL) (Roche), 4 µL RNase A (100 mg/mL) (Qiagen), and 171 µL PBS (phosphate-buffered saline, Sigma-Aldrich), and incubated at 65°C for 20 minutes. Bacterial DNA was extracted using a MagNA Pure Compact Nucleic Acid Isolation kit (Roche), which is based on magnetic bead technology, using the DNA Bacteria v3_2 protocol. The total volume of extracted DNA was 50 µL. The quality and quantity of purified DNA was assessed using the Qubit® dsDNA Broad Range (BR) Assay Kit at the Qubit® 3.0

Fluorometer (Life Technologies, Paisley, UK), and NanoDrop 2000 (Thermo Scientific, UK). The input DNA for library preparation was 1 ng in total.

A library was prepared using the Nextera XT DNA Library Preparation Kit v3 (Illumina), following the manufacturer's protocol. Firstly, genomic DNA was tagmented using Nextera transposome and then tagged with adapter sequences. The tagmented DNA was amplified using a Nextera XT v2 Index Kit set D (Illumina) with Index 1 (N718, 720-724, 726-729) and Index 2 (S513, 515-518, 520-522) adapters for cluster formation. To clean and remove short library fragments of the amplified/tagmented DNA, AMPure XP beads were used in a 3:2 ratio PCR product: volume beads. Freshly diluted 80% ethanol was used for washing, and DNA was eluted in 52.5 µL of the Resuspention Buffer provided by Illumina. In order to assess average fragment size 1 µL of undiluted library was run on the 2200 TapeStation (Agilent Technologies, Santa Clara, USA) using Genomic DNA ScreenTape assay and reagents (Agilent Technologies). The quantity of DNA was measured using a Qubit® dsDNA High Sensitivity (HS) Assay Kit (Life Technologies). Library normalization was performed manually based on the average DNA fragment size and the DNA concentration was adjusted to 4 nM/µL. Subsequently 5 µL of the individual normalized 4nM library was pooled in one tube. Then 5 µL of pooled library was denatured with 5 µL of 0.2 M NaOH and 990 µL of prechilled hybridization buffer (HT1) were added, resulting in a 20 pM denatured library which was later diluted to 10 pM. PhixX control (20 pM) provided by Illumina was prepared according to the manufacture's protocol, and 594 µL of denatured library together with 6 µL Phix control were uploaded to the sequencing flow cell (MiSeq Reagent Kit v3, Illumina) and paired-end sequenced for 2 x 300 cycles.

2.2.3.8 β-Lactamase gene identification in cultivated isolates

Bacterial identification and β-lactamase profiling from Illumina sequences were performed with Illumina Basespace using Kraken Metagenomics version 1.0.0 software and SRST2 version 0.1.5 software respectively. Both applications used Illumina data in fastq format as an input, which were then uploaded into Basespace.

Species identification were BLAST searched against the MiniKraken 20141208 database. The output was presented as a report and visualized in Krona chart, which included the percentage and number of reads covered by the clade rooted at the NCBI taxonomy ID. β-Lactamase genes were sought on the SRST2 (Short Reads Sequencing Typing) using ARG-Annot database (Antibiotic Resistance Gene Annotation) (Gupta *et al.*, 2014) and the scripts supplied with the application. Briefly, fastq reads were aligned to reference sequence using bowtie2 (version 2.2.4) and each alignment processed using SAMtools (0.1.18) to generate a mpileup file. Parsed mpileup file were used to determinate percent coverage, divergence and mismatches to calculate a score for each possible allele with > 90% identity.

2.3 Multiplex tandem PCR (MT-PCR) for detection of bacterial resistance genes in infected urines and isolates

Multiplex, tandem PCR (MT-PCR) was performed using three sequentially-developed commercial assays: (i) Easy-Plex (8-Plex), (ii) High-Plex (16-Plex), (iii) Easy-Plex (24-Plex) (all AusDiagnostics Pty. Ltd., Sydney, Australia). The technology of these assays was based on two PCR steps allowing the detection of multiple targets in one sample using nested primer pairs. The first step involves pre-amplification of multiple targets using specific primers and processes the PCR for only 15 cycles to minimize competition between individual amplicons. The amplified product are then diluted into reaction tubes containing 'bacterial resistance gene-specific' nested primers, and used as a template in the second real-time PCR step. Step one was performed in the Easy-Plex liquid-handling system (AusDiagnostics Pty. Ltd.) (Figure 18) for pre-amplification, and then samples were automatically diluted and transfered into the step 2 reaction tubes, which were then manually transferred into the real-time PCR instrument (Easy-Plex or High-Plex) provided by AusDiagnostic (Figure 19). Amplification was tracked in real-time, with the product melting temperature (T_m) determined to confirm product identity.

Data analysis was performed automatically using the integrated Easy-Plex Result software (AusDiagnostics) which compared the melting temperature, purity and

quantity of the product against predetermined, expected threshold values. Quantitative analysis of the product was performed by comparison with an internal control (SPIKE) containing ~ 10,000 copies of a synthetic oligonucleotide template with a corresponding primer set. This internal standard acted also as a control to confirm that there was no sample-mediated inhibition of the PCR.

The following reagents and plastic were used in all assays: (i) Medium Mastermix (step 1), (ii) Low Mastermix (step 2), (iii) water and oil tubes (for covering PCR mixtures) placed onto reagent block (see Figure 18), (iv) strip tube containing step 1 multiplexed primers placed onto thermal cycler and 96-dilution plate. Variations involved using reaction tubes in the different formats containing the lyophilized step 2 primers e.g. a 72 well rotor-disc for 8-Plex assay to test a maximum of 9 samples/run; 384 well-plates for the 16-Plex assay with a maximum 24 samples/run; 3 x 8-tube strips for 24-Plex with a maximum 4 samples/run.

Samples (isolates and clinical urines) included in the multiplex tandem PCR assays were selected based on phenotypic testing results or sequencing, with the latter approach aiming to find all antibiotic resistance genes in the particular assays.

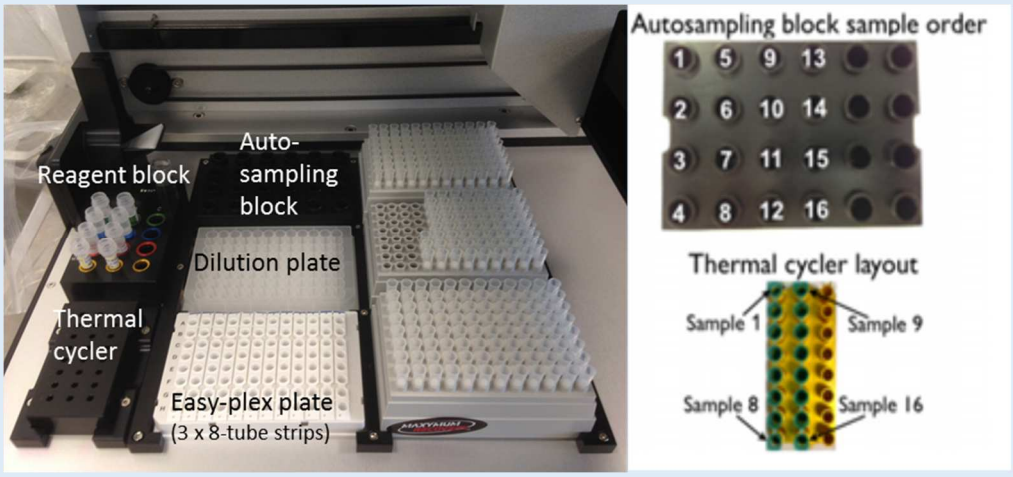


Figure 18. The Easy-Plex™ liquid-handling system with setup processor deck.

Figure 19. The instruments used to perform multiplex-tandem real-time PCR. Legend: The Easy-Plex™ liquid-handling system on the left and real-time PCR instrument (High-Plex) on the right.

2.3.1 Easy-Plex (8-Plex) assay for antibiotic resistance gene detection

The Easy-Plex UTI assay was developed to seek 8 antibiotic resistance genes (Table 7). Three multiplex, tandem PCR assays were run, each using bacteria in four different formats: (i) UTI 1- DNA extracted as below from reference bacterial strains (n=6) and one clinical urine; (ii) UTI 2- Pure cultures from reference isolates taken directly from plates, re-suspended in water and denaturated by heating at 95^{o}C for 4 minutes (n=7); (iii) UTI 3- urine spiked with reference strains to a final density 10^{8}-10^{9} cfu/mL (n=5), (iv) UTI 4- clinical urines from the NNUH (n=2).

For the UTI 1 method pure colonies from overnight CLED plate were re-suspended into 200 µL of lysis buffer (Roche), 180 µL of PBS (Sigma-Aldrich) and 20 µL of proteinase K (20 mg/mL) (Roche). The mixture was incubated for 10 min at 65°C. Bacterial DNA was subsequently extracted using the MagNA Pure Compact Nucleic Acid Isolation Kit (Roche) using the DNA Bacteria v3_2 protocol. The total volume of extracted DNA was 50 µL and the quantity of DNA was assessed according to Qubit (Life Technologies).

Gene identifications were compared with sequencing results from PHE, except for the two clinical urines where the phenotypic comparison was susceptibility testing based on the BSAC disc diffusion methodology at the NNUH.

Table 7. Target genes sought in the 8-Plex assay.

	Target Genes	Enzyme name	Resistances conferred
1.	*dfrA1*	Dihydrofolate reductase DHFR	Trimethoprim
2.	*dfrA5/A14*		
3.	*dfrA12*		
4.	*dfrA7/A17*		
5.	*aac(6')-Ib* (including: Ic, Ig, Iy,Iq II, IIc)	Acetyltransferase AAC(6')-I	Aminoglycoside (amikacin, tobramycin)
6.	*aadA* (including: *aadA1/A2/A3*)	Adenyltransferase ANT(3'')-I	Aminoglycoside (streptomycin)
7.	gyrA1 (including *gyrA1/S, gyrA1/R*)	Gyrase	Fluoroquinolone
8.	*KPparC*	Topoisomerase IV	
	SPIKE Internal Control		

2.3.2 High-Plex (16-Plex) assay for antibiotic resistance genes detection

The High-Plex UTI panel sought 16 antibiotic resistance genes (Table 8). Assays were performed on 74 infected urines from the NNUH, and 35 cultivated isolates from PHE and NNUH, without DNA extraction. Human cells were removed from clinical urines (1-1.5 mL) by centrifugation at 300 *g* for 2 min and then bacterial pellets were collected by 5 min centrifugation at 12,500 *g.* Cultivated isolates together with bacterial pellet from urines were re-suspended in 300 µL of water and denaturated by heat at 95^{o}C for 4 minutes. The resulting suspensions were diluted 10x in water, and then 10 µL was used as a template for the High-Plex system. Depending on the organism, results were compared with Illumina sequencing performed at PHE or

MinION sequencing as described in Section 2.4, real-time SybrGreen PCR (Section 2.3.6), or phenotypic susceptibility testing (BSAC methodology).

Table 8. Target genes sought in the 16-Plex assay.

	Target Gene	Alleles sought (AusDiagnostics data)
β-Lactamases		
1.	*bla*$_{TEM}$	including: 1, 3, 10, 104-106, 71, 76-84, 138, 143, 150, 155
2.	*bla*$_{SHV}$	including: 1, 2, 5,11, 12, 25, 26, 38, 56
3.	*bla*$_{CTX-M\ group\ 1}$	including: 1, 3, 15, 28, 29, 32, 36, 58, 79, 103
4.	*bla*$_{CTX-M\ group\ 9}$	including: 9, 13, 14, 24, 27, 38
5.	*bla*$_{CMY}$	including: 2, 4, 16, 31, 73, combined with assay to detect CMY-1
6.	*bla*$_{OXA-1}$	including: 1, A1, 4, 30
7.	*bla*$_{OXA-48}$	Including 48, 163, 162, 181, 204, 244, 245, 247, 370, 405
8.	*bla*$_{KPC}$	including: 1, 2, 3
9.	*bla*$_{NDM}$	including: 1, 2, 3, 4, 5, 6,7, 8
10.	*bla*$_{VIM}$	including: 1, 2, 3
Trimethoprim resistance determinants		
11.	*dfr*	including: A1, A5/A14
12.	*dfr*	including: A12,A7/ A17
Aminoglycoside resistance determinants		
13.	*aac(6')-Ib*	including: Ic, Ig, Iy,Iq II, IIc, and aac(6')-Ib-cr
14.	*aadA*	including: aadA1, aadA2, aadA3
Fluoroquinolone resistance mutations		
15.	*gyrA1*	including: *gyrA1/S, gyrA1/R*
16.	*KPparC*	
	Pan-bacterial I/D	
	SPIKE (Internal control)	

2.3.3 Easy-Plex (24-Plex) assay for antibiotic resistance gene detection and bacterial genus identification

The High-Plex UTI panel was designed to seek 24 target genes, including the 16 represented in the 16-Plex assay (Table 8). In addition the aminoglycoside resistance panel was expanded to seek four further genes (*aadB, aacC1, aacC2, aacC3*) commonly responsible for gentamicin and/or tobramycin resistance. Additional targets were added to identify non Enterobacteriaceae bacteria to genus level (pan-*Enterococcus*, pan-*Streptococcus*, pan- *Staphylococcu*s, pan- *Pseudomonas*).

The assay was performed directly on 23 samples (16 infected clinical urines and 7 bacterial isolates including 4 reference isolates from PHE). The template for the Easy-Plex assay was prepared similarly as for the 16-Plex assay. Firstly, human cells were removed from clinical urine (1.5 mL) by centrifugation at 300 *g* for 2 min. Bacterial pellets were then collected through 5 min centrifugation at 12,500 *g* and re-suspended in water (100 µL-1 mL) before being denaturated by heating at 95^{o}C for 4 minutes. Isolates from fresh overnight cultures were similarly re-suspended and denaturated. The resulting suspensions were diluted 10x in water and then 10 µL was used as a template for the Easy-Plex system.

Data analysis from the 24-Plex assay was performed on the EasyPlex Result software version 1.6.4 provided by AusDiagnostics and updated with an algorithm to guide antibiotic choice for treatment. Interpretations and advice were predicated upon the guide presented in Table 9. Example outputs were e.g.:

(i) "No OXA-48, No KPC, No VIM/NDM, No CMY-1, No CTX-M group 1, No CTX-M group 9, No TEM, No SHV, No OXA 1"; "Patient may respond to oral Ampicillin or amoxicillin or oral cephalexin, only if *E. coli* or *P. mirabilis* were detected".

(ii) "No dfrA1/A5, No dfrA12/A17"; "Patient may respond to oral trimethoprim".

Results were compared with Illumina sequencing at PHE and UEA as described in Section 2.4 (for cultivated isolates), real-time SybrGreen PCR (Section 2.3.6) and phenotypic susceptibility testing.

Table 9. AusDiagnostics algorithm to aid result interpretation and to guide therapy with the 24-Plex software.

(A) Treatment options *vs*. β-lactamase producing Enterobacteriaceae with classical penicillinases, AmpC and ESBLs enzymes.

First line treatment for community-acquired UTI (uncomplicated UTI)	**Gene detected**					**Comments**
	***bla*TEM**	***bla*SHV**	***bla*CTX-M gr1**	***bla*CTX-M gr9**	***bla*CMY**	
Ampicillin or Amoxicillin	R	R	R	R	R	Mechanism is generally associated with resistance to this agent
Cephalexin	P	P	R	R	R	Mechanism MAY confer resistance depending on level of expression, particular enzyme variant, of other strain characteristics. Base use (or not) on phenotypic results and local resistance patterns Mechanism is generally associated with resistance to this agent
Trimethoprim[1]	P	P	P	P	P	Mechanism does not confer resistance to this agent
Nitrofurantoin[2]	S	S	S	S	S	Mechanism does not confer resistance to this agent
Fosfomycin[3]	S	S	S	S	S	Mechanism does not confer resistance to this agent
Pivmecillinam	S	P	P	P	S	Mechanism does not confer resistance to this agent Mechanism MAY confer resistance depending on level of expression, particular enzyme variant, of other strain characteristics. Base use (or not) on phenotypic results and local resistance patterns
Amoxicillin-clavulanic acid[4]	P	P	P	P	R	Mechanism MAY confer resistance depending on level of expression, particular enzyme variant, of other strain characteristics. Base use (or not) on phenotypic results and local resistance patterns Mechanism is generally associated with resistance to this agent
Ciprofloxacin[5]	P	P	P	P	P	Mechanism does not confer resistance to this agent

Second line treatment for hospital-acquired UTI (complicated UTI)	***bla*TEM**	***bla*SHV**	***bla*CTX-M gr1**	***bla*CTX-M gr9**	***bla*CMY**	**Comments**
3rd generation cephalosporin	P	P	R	R	R	Mechanism MAY confer resistance depending on level of expression, particular enzyme variant, of other strain characteristics. Base use (or not) on phenotypic results and local resistance patterns Mechanism is generally associated with resistance to this agent
Piperacillin/Tazobactam	P	P	P	P	R	Mechanism MAY confer resistance depending on level of expression, particular enzyme variant, of other strain characteristics. Base use (or not) on phenotypic results and local resistance patterns Mechanism is generally associated with resistance to this agent
Gentamicin	P	P	P	P	P	Mechanism does not confer resistance to this agent
Tobramycin	P	P	P	P	P	Mechanism does not confer resistance to this agent
Amikacin	P	P	P	P	P	Mechanism does not confer resistance to this agent
Carbapenem	S	S	S	S	S	Mechanism does not confer resistance to this agent
Colistin[6]	P	P	P	P	P	Mechanism does not confer resistance to this agent

(B) Treatment options *vs*. β-lactamase genes producing in Enterobacteriaceae with classical penicillinases and carbapenemases.

First line treatment for Community-acquired UTI (uncomplicated UTI)	**Gene detected**					**Comments**
	***bla*$_{OXA-1}$**	***bla*$_{OXA-48}$**	***bla*$_{KPC}$**	***bla*$_{VIM}$**	***bla*$_{NDM}$**	
Ampicillin or Amoxicillin	R	R	R	R	R	Mechanism is generally associated with resistance to this agent
Cephalexin	P	R	R	R	R	Mechanism does not confer resistance to this agent Mechanism is generally associated with resistance to this agent
Trimethoprim[1]	P	P	P	P	P	Great majority of the carbapenemase strains (OXA-48, KPC, VIM, NDM) are multi-resistant. Treatment mostly bases on the susceptibility test results. Mechanism does not confer resistance to this agent
Nitrofurantoin[2]	P	P	P	P	P	Great majority of the carbapenemase strains (OXA-48, KPC, VIM, NDM) are multi-resistant. Treatment based on the susceptibility test results. Mechanism does not confer resistance to this agent
Fosfomycin	P	P	P	P	P	Great majority of the carbapenemase strains (OXA-48, KPC, VIM, NDM) are multi-resistant. Treatment based on the susceptibility test results. Mechanism does not confer resistance to this agent
Pivmecillinam	P	R	R	R	R	Mechanism does not confer resistance to this agent Mechanism is generally associated with resistance to this agent
Amoxicillin-clavulanic acid[4]	R	R	R	R	R	Mechanism is generally associated with resistance to this agent
Ciprofloxacin[5]	P	P	P	P	P	Great majority of the carbapenemase strains (OXA-48, KPC, VIM, NDM) are multi-resistant. Treatment based on the susceptibility test results. Mechanism does not confer resistance to this agent

Second line treatment for hospital-acquired UTI (complicated UTI)	*bla*$_{OXA-1}$	*bla*$_{OXA-48}$	*bla*$_{KPC}$	*bla*$_{VIM}$	*bla*$_{NDM}$	Comments
3rd generation cephalosporin	P	P	R	R	R	Great majority of the carbapenemase strains (OXA-48, KPC, VIM, NDM) are multi-resistant. Treatment based on the susceptibility test results. Mechanism does not confer resistance to this agent Mechanism is generally associated with resistance to this agent
Piperacillin/Tazobactam	P	R	R	R	R	Great majority of the carbapenemase strains (OXA-48, KPC, VIM, NDM) are multi-resistant. Treatment based on the susceptibility test results. Mechanism MAY confer resistance depending on level of expression, particular enzyme variant, of other strain characteristics. Base use (or not) on phenotypic results and local resistance patterns Mechanism is generally associated with resistance to this agent
Gentamicin	P	P	P	P	R	Great majority of the carbapenemase strains (OXA-48, KPC, VIM, NDM) are multi-resistant. Treatment based on the susceptibility test results. Mechanism does not confer resistance to this agent Mechanism is generally associated with resistance to this agent
Tobramycin	P	P	P	P	R	Great majority of the carbapenemase strains (OXA-48, KPC, VIM, NDM) are multi-resistant. Treatment based on the susceptibility test results. Mechanism does not confer resistance to this agent Mechanism is generally associated with resistance to this agent

Second line treatment for hospital-acquired UTI (complicated UTI)	*bla*$_{OXA-1}$	*bla*$_{OXA-48}$	*bla*$_{KPC}$	*bla*$_{VIM}$	*bla*$_{NDM}$	Comments
Amikacin	P	P	P	P	R	Great majority of the carbapenemase strains (OXA-48, KPC, VIM, NDM) are multi-resistant. Treatment based on the susceptibility test results.
						Mechanism does not confer resistance to this agent
						Mechanism is generally associated with resistance to this agent
Carbapenem	P	R	R	R	R	Mechanism MAY confer resistance depending on level of expression, particular enzyme variant, of other strain characteristics. Base use (or not) on phenotypic results and local resistance patterns
						Mechanism is generally associated with resistance to this agent
Colistin[6]	S	S	S	S	S	Mechanism does not confer resistance to this agent

(C) Treatment options *vs*. aminoglycoside and trimethoprim genes detected in Enterobacteriaceae

First line treatment for Community-acquired UTI (uncomplicated UTI)	**Gene detected**			**Comments**
	aac(6')-1b	***aadA1/A2/A3***	***dfrA1/A5/A12/A17***	
Ampicillin or Amoxicillin	P	P	P	Mechanism does not confer resistance to this agent
Cephalexin	P	P	P	Mechanism does not confer resistance to this agent
Trimethoprim[1]	P	P	R	Mechanism does not confer resistance to this agent
				Mechanism is generally associated with resistance to this agent
Nitrofurantoin[2]	S	S	S	Mechanism does not confer resistance to this agent
Fosfomycin[3]	S	S	S	Mechanism does not confer resistance to this agent
Pivmecillinam	S	S	S	Mechanism does not confer resistance to this agent
Amoxicillin-clavulanic acid[4]	P	P	P	Mechanism does not confer resistance to this agent
Ciprofloxacin	R	P	P	Mechanism MAY confer resistance depending on level of expression, particular enzyme variant, of other strain characteristics. Base use (or not) on phenotypic results and local resistance patterns
				Mechanism does not confer resistance to this agent
Second line treatment for hospital-acquired UTI (complicated UTI)	***aac(6')-1b***	***aadA1***	***dfrA1/A5/A12/A17***	**Comments**
3rd generation cephalosporin	P	P	P	Mechanism does not confer resistance to this agent
Piperacillin/Tazobactam	P	P	P	Mechanism does not confer resistance to this agent
Gentamicin	P	P	P	Mechanism does not confer resistance to this agent
Tobramycin	R	P	P	Mechanism is generally associated with resistance to this agent
				Mechanism does not confer resistance to this agent
Amikacin	R	P	P	Mechanism is generally associated with resistance to this agent
				Mechanism does not confer resistance to this agent
Carbapenem	S	S	S	Mechanism does not confer resistance to this agent
Colistin[7]	S	S	S	Mechanism does not confer resistance to this agent

Legend:

R- Resistance likely to be conferred by gene found

P- Resistance may be conferred by gene found, depending on level of expression

S- Not compromised by gene found

1 *dfrA1/A5/A12/A17* genes encode the dihydrofolate reductases most often responsible for trimethoprim resistance; if they are absent, assess trimethoprim treatment based on the susceptibility test results. Inherent resistance in *Pseudomonas* spp.

2Appropriate only for uncomplicated lower UTI caused by Enterobacteriaceae.
Inherent resistance in Proteeae (*Proteus* spp., *Morganella* spp*., Providenicia* spp.)

3 Oral formulation appropriate only for uncomplicated lower UTI caused by Enterobacteriaceae.
Not suitable (as an oral agent) for pyelonephritis or severe urinary infection due to low systemic levels.

4 Not active vs. *Citrobacter freundii*, *Enterobacter* spp., *Serratia* spp., *Morganella* spp., *Providenicia* spp., *Pseudomonas aeruginosa.*

5 Resistance is unlikely when the melting temp of the '*Enterobacteriaceae'* gene = 83.5^{o}C and that for *gyr A1/S* = 86-87^{o}C as this implies ciprofloxacin-susceptible *E. coli*. For other coliform species base treatment on phenotypic susceptibility test results and local epidemiology.

6 Reserve for use against multi-drug resistant Gram-negative bacteria.
Not suitable for the infection caused by Proteeae (*Proteus* spp., *Morganella* spp*., Providenicia* spp.)

2.3.4 *Phenotypic susceptibility characterisation of uropathogens*

Reference isolates from PHE and those recovered from clinical urines were grown on CLED media (Becton Dickinson) at 37^{o}C overnight. Species were identified on MALDI-TOF. *In vitro* susceptibility testing was performed according to the BSAC disc diffusion method using commercially available disc types from Oxoid (Table 4).

2.3.5 *Genomic sequences for antibiotic resistance genes identification*

Bacterial DNA from cultivated isolates (n=12) was used as a template to evaluate the 8-Plex assay. These organisms had been previously sequenced by Illumina methodology at PHE as described in Section 2.4.8. Bacterial DNA from 19 out of 35 cultivated isolates used in the 16-Plex performance and 8 cultivated isolates run at 24-Plex assay also were sequenced either (by myself) on MinION or Illumina at PHE see Section 2.4 or on Illumina (by myself) at UEA as presented in Section 2.2.3.7.

2.3.6 *Real time SybrGreen PCR for antibiotic resistance genes detection*

Real time SybrGreen PCR, with specific primers was used to detect genes for classical penicillinases or their ESBL variants (*bla*$_{TEM,}$ *bla*$_{OXA-1,}$ *bla*$_{SHV}$), cephalosporinases (*bla*$_{CMY,}$) and extended-spectrum β-lactamases (*bla*$_{CTX-M\ gr-1}$, *bla*$_{CTX-M\ gr-9,}$) to compare results from the 16-and 24-Plex assays.

Primers were designed using Primer3Plus software (http://primer3plus.com) and are listed in Table 10. Real-time PCR was performed using the LightCycler 480 (Roche) with a final volume of 20 µL. Each reaction contained 2x Sybr Green I mastermix, 10 pM primer forward (F) and reverse (R) and water. A single colony from the overnight culture was resuspnded in 100 µL of water, denaturated at 95°C for 4 min and used as a template (2 µL). The PCR programme consisted of an initial denaturation step at 95°C for 5 min, followed by 35 cycles of DNA denaturation at 95°C for 20 s, primer annealing at 60°C for 20 s, and primer extension at 72°C for 30

s. After the last cycle, the melting curve analysis was followed by denaturation at 95°C, then cooling to 72°C. Fluorescence signals were collected at 530 nm wavelength continuously from 72°C to 99°C at 0.2°C per second.

Table 10. Primers sequenced used for SybrGreen assay.

Primers	Primers sequence (5'-3')	GenBank	Product size (bp)	Allels detected
TEM_F	CAGCGGTAAGATCCTTGAGAG	KU376497.1	326	1, 3, 10, 104-106, 71, 76-84, 138, 143, 150, 155
TEM_R	GAGTTACATGATCCCCCATGTT			
SHV_F	CGCCTGTGTATTATCTCCCTGT	EU586041.1	316	1,2, 11, 25, 26, 38, 56
SHV_R	CAAGGTGTTTTTCGCTGACC			
CMY-F	GAGTTACGAAGAGGCAATGACC	GQ351345.1	310	2, 4, 16, 31, 73
CMY_R	CCAGCCTAATCCCTGGTACATA			
OXA1_F	AGACGTGGATGCAATTTTCTGT	J02967.2	319	1, A1, 4, 30
OXA1_R	GCACCAGTTTTCCCATACAGTT			
CTX-M gr1_F	GCAAAAACTTGCCGAATTAGAG	AJ549244.1	320	1, 3, 15, 28, 29, 32, 36, 58, 79, 103
CTX-M gr1_R	GCTTATTCATCGCCACGTTATC			
CTX-M gr9_F	CTTTCCAATGTGCAGTACCAGT	AF252623.2	320	9, 13, 14, 24, 27, 38
CTX-M gr9_R	CGGTATTCAGCGTAGGTTCAG			

2.4 MinION Nanopore sequencing

Specimens tested by MinION nanopore sequencing were selected based on culture results, with a bias towards multiply resistant strains to test the ability to detect various antibiotic resistance genes.

For this purpose ten heavily infected (>10^7 cfu/mL) clinical urines from the Microbiology Department of the NNUH were tested. Additionally to seek a diversity of antibiotic resistance genes, four uninfected urines from a healthy volunteer were spiked with 10^8 cfu/mL of multi-drug resistant *E. coli* strain H141480453, which produced NDM-4 and OXA-181 carbapenemases, CTX-M-15 extended-spectrum β-lactamase and CMY-2 AmpC β-lactamase or with the *E. coli* strain recovered from Clinical Urine 6.

2.4.1 Sample preparation for MinION Nanopore sequencing

In order to obtain the maximum recovery of bacterial DNA from urine and minimize contamination with human DNA methodology was refined progressively during the project. In the final, optimised procedure clinical urine (4-10 mL) was centrifuged at 2,000 rpm (300 *g*) for 2 min to remove human cells. The supernatant was collected and re-centrifuged at 13,500 rpm for 5 min. The resulting bacterial pellet was re-suspended in 1 mL of PBS (Sigma-Aldrich), and treated with the MolYsis Basic 5 Kit (MolYsis Life Science, Bremen, Germany), as described in the manufacture's instructions to lyse residual human cells and remove their DNA. Then, 280 µL of lysis buffer and 20 µL of proteinase K (20 mg/mL) (Roche) were added to lyse the bacteria, and the final mixture was incubated for 10 min at 65°C. Bacterial DNA was subsequently extracted using the MagNA Pure Compact Nucleic Acid Isolation Kit (Roche) with the DNA Bacteria v3_2 protocol. The total volume of extracted DNA was 50 µL.

Methodology evolved in the course of the study (see Table 11), and the MinION system itself was also refined. Early variations of the method were: (i) the initial centrifugation was omitted and no human DNA depletion step was performed for

Clinical Urine 1; and (ii) the NEBNext® Microbiome DNA Enrichment Kit (New England BioLabs, Hitchin, UK) was used to bind and remove double-stranded methylated human DNA following the manufacture's procedure, with 380 µL of lysis buffer added, for Clinical Urines 2-4.

To spike urines, 1 mL of overnight broth culture (10^9 cfu/mL) was added to 9 mL of uninfected midstream donor urine, 4 mL of which was then used for DNA extraction, always with the final version of the sample preparation methodology.

The quality and concentration of the recovered DNA was assessed using a Qubit® 2.0 Fluorometer (Life Technologies) and 2200 TapeStation (Agilent Technologies); DNA concentrations >15 mg/L were considered acceptable (i.e. the minimum starting material for sequencing was 750 ng).

2.4.2 MinION library preparation with SQK-MAP-002-005 Nanopore Sequencing Kit

To generate a library with an average fragment size of 8 kb, DNA (750 ng –-2 µg) was fragmented into a G-tube (Covaris, Brighton, UK) and centrifuged at 7,200 rpm (3600 *g*) for 1 minute before inverting the tube and centrifuging again with the same conditions. The sample was additionally pulse-centrifuged at 4,000 *g* if the liquid had not completely passed through the ruby orifice. The library preparation procedure was refined during the study. Variations, in earlier library preparation experiments are presented in Table 11. In particular: (i) Kit SQK-MAP-002 was used for Clinical Urines 1-4, following the protocol described by (Quick *et al.*, 2014); (ii) Kit SQK-MAP-003 was used with Clinical Urines 5-6 and for Run 1 with urine spiked with *E. coli* H141480453, again following the protocol described by (Quick *et al.*, 2014); (iii) Kit SQK-MAP-004 and the procedure of (Urban *et al.,* 2015) was used with Clinical Urine 7 and urine spiked with the *E. coli* isolate recovered from Clinical Urine 6, (iv) Kit SQK-MAP-005 was used with Clinical Urines 8-10 and second run of urine spiked with *E. coli* H141480453, following the method of (Ip *et al.*, 2015). After fragmentation a PreCR repair step (New England BioLabs, Hitchin, UK), aiming to repair damaged template DNA, was performed following the manufacturer's protocol for Clinical

Urines 5-10, and three 'spike urines', two of them with *E. coli* strain H141480453 and one with the *E. coli* recovered from Clinical Urine 6.

The reaction product (100 µL) was cleaned with 1 x Agencourt AMPure XP Beads (Beckman Coulter, High Wycombe, UK) at a ratio of 1 part beads: 1 part reaction mixture. These mixtures were incubated on a magnetic rack (Invitrogen MagnaRack) for 3 min and washed twice in 200 µL of freshly prepared 70% ethanol while still on the magnet, and then eluted into 81 µL of 10 mM Tris-HCl pH 8.5. An end-repair step was then performed using the NEBNext® End Repair Module (NEB) according to manufacturer's instruction and the resulting blunt-ended DNA (100 µL) was cleaned using 1 x Agencourt AMPure XP Beads, and eluted in 26 µL of 10 mM Tris-HCl pH 8.5. To prevent concatamer formation during library preparation, 3'-dA DNA tailing was then performed using the NEBNext dA-Tailing Module (NEB), as described in the producer's instructions. For the samples that were run with Genomic DNA Sequencing Kit SQK-MAP-005 only (CUs 8-10, and the second run of urine spiked with *E. coli* H141480453) the reaction product (30 µL) was cleaned with 1 x Agencourt AMPure XP Beads.

The Genomic DNA Sequencing Kit (Oxford Nanopore Technologies, Oxford, UK) was used to generate a MinION sequencing library. Briefly, adenylated DNA and Blunt/TA ligase master mix (T4 DNA ligase, New England Biomolecular) were added to the reagents from Genomic DNA Sequencing Kit and the reaction was left for 10 minutes at room temperature. AMPure XP Beads (SQK-MAP002 - 003) or His-Tag Dynabeads (SQK-MAP004 - 005) (Life Technology, Paisley, UK) were then used to clean the adapter-ligated DNA and eluted in 26 µL of Elution Buffer (Oxford Nanopore). After each clean-up step 1 µL volumes of eluted sample were used to quantify DNA on a Qubit® 2.0 Fluorometer (Life Technologies).

Table 11. Early versions of MinION library preparation procedure.

	CUs 1-4	CUs 5-6, SU Run 1	CU 7, SCU 6	CUs 8-10, SU Run 2
Step	**SQK-MAP002**	**SQK-MAP003**	**SQK-MAP004**	**SQK-MAP005**
1	**Fragmentation** using *g*-Tube 7,200 rpm-1 min	**Fragmentation** using *g*-Tube 7,200 rpm -1 min	**Fragmentation** using *g*-Tube 7,200 rpm-1 min	**Fragmentation** using *g*-Tube 7,200 rpm-1 min
2	PreCR was not performed	**PreCR** 37°C-30 min	**PreCR** 37°C-30 min	**PreCR** 37°C-30 min
3	Clean-up was not performed	**Clean-up** using Agencourt AMPure XP Beads Elution in 80 µL of molecular grade water	**Clean-up** using Agencourt AMPure XP Beads Elution in 80 µL of 10 mM Tris-HCl pH 8.5	**Clean-up** using Agencourt AMPure XP Beads Elution in 80 µL of 10 mM Tris-HCl pH 8.5
4	**End-repair** Room temp-30 min	**End-repair** Room temp-30 min	**End-repair** Room temp-30 min	**End-repair** Room temp-20 min
5	**Clean-up** using Agencourt AMPure XP Beads Elution in 25 µL of molecular grade water	**Clean-up** using Agencourt AMPure XP Beads Elution in 25 µL of molecular grade water	**Clean-up** using Agencourt AMPure XP Beads Elution in 25 µL of 10 mM Tris-HCl pH 8.5	**Clean-up** using Agencourt AMPure XP Beads Elution in 25 µL of 10 mM Tris-HCl pH 8.5
6	**dA-tailing** 37°C-30 min	**dA-tailing** 37°C- 30 min	**dA-tailing** 37°C- 30 min	**dA-tailing** 37°C- 10 min
7	Clean-up was not performed	Clean-up was not performed	Clean-up was not performed	**Clean-up** using Agencourt AMPure XP Beads Elution in 30 µL of 10 mM Tris-HCl pH 8.5
8	**Adapter Ligation**	**Adapter Ligation** containing tether and hairpin motor	**Adapter Ligation** containing tether and hairpin motor	**Adapter Ligation** containing tether and hairpin motor
9	**Clean-up** using Agencourt AMPure XP Beads Elution in 25 µL of Elution Buffer provided by ONT	**Clean-up** using Agencourt AMPure XP Beads with bovine serum albumin (BSA- 50 mg/mL) Elution in 25 µL of Elution Buffer provided by ONT containing tether	**Clean-up** using His-Tag Dynabeads Elution in 25 µL of Elution Buffer provided by ONT containing tether	**Clean-up** using His-Tag Dynabeads Elution in 25 µL of Elution Buffer provided by ONT containing tether
10	**Tether attachment** Room temp-10 min	Tether attachment was not performed	Tether attachment was not performed	Tether attachment was not performed

	CUs 1-4	CUs 5-6, SU Run 1	CU 7, SCU 6	CUs 8-10, SU Run 2
Step	**SQK-MAP002**	**SQK-MAP003**	**SQK-MAP004**	**SQK-MAP005**
11	**Hairpin motor attachment** Room temp-overnight incubation	Hairpin motor attachment was not performed	Hairpin motor attachment was not performed	Hairpin motor attachment was not performed
12	**Flow cell preparation** Platform QC run Run twice with 150 µL of EP Buffer (provided by ONT) – 10 min	**Flow cell preparation** Platform QC run Run twice with 147 µL of EP Buffer and 3 µL of Fuel Mix (provided by ONT)	**Flow cell preparation** Platform QC run Run twice with 147 µL of EP Buffer and 3 µL of Fuel Mix (provided by ONT)	**Flow cell preparation** Platform QC run Run twice 75 µL of Running Buffer, 66 µL water and 3 µL of Fuel Mix (provided by ONT)
13	Loading Pre-sequencing mix	Loading Pre-sequencing mix	Loading Pre-sequencing mix	Loading Pre-sequencing mix

2.4.3 MinION Library Preparation with SQK-MAP-006 Nanopore Sequencing Kit

Fragmentation was performed as described in Section 2.3.2 for SQK-MAP-002-005. After fragmentation, repair was done for 15 min at 20^{o}C with the NEBNext FFPE DNA Repair Mix (NEB), used according to the manufacturer's instructions. The reaction product (62 µL) was cleaned as previously described (Section 2.3.2) with 1 x Agencourt AMPure XP Beads. DNA was eluted into 46 µL of 10 mM Tris-HCl pH 8.5. End Repair/dA-tailing using a NEBNext Ultra II End Repair/dA-tailing Kit (NEB) was performed at 20°C for 5 minutes and 65°C for 5 min. The reaction product (60 µL) was cleaned again. Adapter ligation and the tethering step were done using Genomic DNA Sequencing Kit SQK-MAP-006 following the manufacturer's procedure. Genomic DNA Sequencing Kits (Oxford Nanopore Technologies) were next used to generate a MinION sequencing library. With the final version of this kit (SQK-MAP-006), used for Run 3 of urine spiked with *E. coli* H141480453 adenylated DNA and Blunt/TA ligase master mix (T4 DNA ligase, NEB) were used to ligate the hairpin adapter and tether (provided with kit) to the dA- tailed DNA. The reaction was left for 10 min at room temperature. Dynabeads® MyOne™ Streptavidin C1 (Life Technology) were then used to clean the adapter-ligated DNA, which was subsequently eluted in 26 µL of Elution

Buffer (Oxford Nanopore Technologies). A QC (Quality Control) run was performed by twice uploading 500 µL of Running Buffer, Fuel Mix and water mixture into the sample port.

2.4.4 MinION library preparation using 'Rapid Sequencing Kit'

The MinION Rapid Sequencing Kit (ONT), with a 15-minute library preparation procedure was used for Spiked Urine Run 4, the final assay performed by MinION. Instead of shearing in a Covaris *g*-TUBE, genomic DNA was fragmented using the transposase enzyme, which simultaneously attached adapters to the free ends. Y-adapters were then added, but no hairpin adapters. When DNA passed through the pore, only one strand of the duplex was sequenced (1-D reads).

The procedure consists of two steps: tagmentation and ligation. To perform tagmentation 10 µL of DNA (200 ng) was added to 10 µL of FRM reagent (provided by Oxford Nanopore) and incubated at 30 °C for 1 min and at 75 °C for 1 min. Subsequently, 1 µL of RAD reagent (provided by Oxford Nanopore) was added to the tagmented DNA and mixed with 1 µL of Blunt/TA Ligase Master Mix (T4 DNA Ligase, NEB). The sample was then incubated for 5 min at room temperature before loading into the flow cell (5 µL sample, 75 µL Running Buffer, 66 µL nuclease-free water, and 4 µL Fuel Mix).

2.4.5 MinION Sequencing

MinION sequencing was variously performed using R7.0 and R7.3 Chemistry (Oxford Nanopore Technology), with the former used for Clinical Urines 1-4 and the latter for Clinical Urines 5-10 and all spiked urines. A QC run was firstly performed to assess the flow cell's number of active pores as described in Table 11 (step 12). After 10 min, this was followed by Pre-Sequencing Library Preparation mix diluted in EP Buffer/Running Buffer, Fuel Mix and water (Oxford Nanopore Technologies) and, finally, by 150 µL of Sequencing Mix. Sequencing was run for 7.5 - 48 h. Oxford Nanopore Technologies' MinKNOW software was used to collect raw electric signal data, which were base-called using the Metrichor™ Agent software (MinKNOW™ version 0.45.2.6 (R7.0) or MinKNOW™ version 2.34.3 (R7.3).

2.4.6 BLAST and CARD alignment for pathogen identification and resistance gene detection from clinical urine samples and urine spiked with E. coli H141480453 Run 1, 2, and 4

Identification of species and resistance genes routinely utilised BLAST search and the CARD (Comprehensive Antibiotic Resistance Database) database (McArthur *et al.*, 2013). MinION data were extracted, in fasta format, from raw HDF5 files using Poretools (Loman & Quinlan, 2014). Read statistics were collected *via* Biopython and visualised graphically using R software (Watson *et al.*, 2015). To identify the pathogen species, BLAST non-redundant databases were built for 'proteobacteria', 'firmicutes' and 'human'. Single top hits from each of these separate database aliases were identified in a parallel megablast process. Taxa was distinguished using the in-house script blast_separate_taxa.pl, and taxonomy was assigned using blast_taxonomy_report.pl (Kumar *et al.*, 2013) with some modifications as described at https://hithub.com/LCrossman.

Resistance genes were identified by alignment of the MinION reads to the CARD database using LAST, with parameters optimised for low accuracy and long matches (Kiełbasa *et al.*, 2011; Frith *et al.*, 2010b; Frith *et al.*, 2010a). Some sequences in CARD contain resistance-gene-flanking regions, which can lead to false positive resistance results, therefore matches were verified to ensure that reads were not exclusively mapping to flanking regions by direct visualization in Artemis (Sanger) and by examination of the coordinates. Consensus sequences were produced based upon the CARD database reference sequences using the MinION read alignments by Samtools 0.1.19, the Samtools mpileup workflow, bcftools, vcfutils.pl and vcf2fq, ultimately generating indexed Bam files (Li *et al.*, 2009).

BLASTn (BLAST v 2.2.30+) top hits were identified, using consensus sequences, against the CARD database, seeking >80% identity over the full length of a gene. In addition, reciprocal BLAST best-hits were identified between the consensus sequences and the CARD database. The resulting output data were parsed and sorted with a final report generated by a Python script.

2.4.7 WIMP and ARMA alignment for pathogen identification and resistance gene detection from urine spiked with E. coli H141480453 Run 3

Oxford Nanopore Technologies' WIMP and ARMA applications were released toward the end of this study, and were tested for Spiked Urine Run 3 with *E. coli* H141480453.

'What's in my pot?' (WIMP) (Juul *et al.* 2015) software on the Metrichor platform identifies the microbial species in real-time, using a reference database and Kraken 11 (Juul *et al.,* 2015). The Antimicrobial Resistance Mapping Application (ARMA, Metrichor) (ONT) is a bioinformatics application from Metrichor Ltd. that allows real-time detection of antibiotic resistance genes from either a mixed or single-species sample of microbes. The application first runs standard 2-D Basecalling from Oxford Nanopore Technologies and then uses the LAST program to align the base-called reads against the CARD database. The antibiotic-resistance ontology (ARO) within CARD describes how the genes are related to antibiotic resistance phenotypes.

2.4.8 Illumina library preparation of cultivated isolates recovered from clinical urines, and E. coli H141480453

This work was done externally and two methods were used to sequence DNA from cultivated bacteria from clinical urine samples, according to whether work was done at PHE or Brunel University.

At PHE's Genomic Services Unit, genomic DNA was prepared using a GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, USA) and sequenced on a HiSeq instrument (Illumina) in Rapid Run mode, using the TruSeq Rapid PE Cluster v2 kit and TruSeq Rapid SBS v2 kits (200 cycles) (Turton *et al.*, 2015). Library preparation was with Nextera XT DNA sample preparation kits (Illumina), following the manufacturer's protocol.

At Brunel University, extracted bacterial DNA was quantified using Quant-iT™ PicoGreen® dsDNA Kits (Life Technology, Paisley, UK) and a FLUOstar OPTIMA plate

scanner (BMG Labtech, Ortenberg, Germany) used according to the manufacturers' specifications. DNA (300 ng) was fragmented using an Episonic (Epigentek, New York, USA) system. Libraries were constructed using the NEBNext Ultra DNA Sample Prep Master Mix Kit (NEB) with minor modifications and a custom automated protocol on a Biomek FX (Beckman Coulter, High Wycombe, UK). Ligation was performed using Illumina Adapters (Multiplexing Sample Preparation Oliogonucleotide Kit) and ligated libraries were size-selected using Agencourt AMPure XP Beads (Beckman). Samples were sequenced on the 150-base paired-end Illumina HiSeq 2000 platform.

2.4.9 CARD alignment for resistance gene detection from cultivated bacteria

The presence of resistance determinants from Illumina sequence reads was determined with 'Genefinder', an in-house Public Health England algorithm that uses bowtie2 (http://bowtie-bio.sourceforge.net/bowtie2) to map the reads to a local database of antimicrobial resistance genes and Samtools version 0.1.18 (http://samtools.sourceforge.net) to generate an mpileup file. The script than parses the mpileup file to define the presence of any reference sequence from the database based on read coverage and nucleotide identity with a threshold of > 90% identity over the full length of the sequence.

2.4.10 Phenotypic characterisation of uropathogens

Bacteria were grown from the urines by standard methodology (PHE, 2016d) and identified by MALDI-TOF mass spectroscopy (Brüker Daltonik GmbH). Minimum inhibitory concentrations (MICs) of antibiotics were determined at Public Health England by British Society for Antimicrobial Chemotherapy agar dilution testing with results categorised according to the breakpoints of the European Committee on Antimicrobial Susceptibility Testing (http://www.eucast.org). Susceptibility testing was also performed at the UEA by the BSAC disc diffusion method using discs from Oxoid, with BSAC breakpoints (http://bsac.org.uk).

Chapter 3

RESULTS

3.1 Epidemiology of UTI specimens at the NNUH

AIMS:

- To describe and understand the local epidemiology and the antimicrobial resistance profiles of urinary tract infection and pathogens at the NNUH.
- To provide understanding of the background to which any rapid diagnostic would need to be applied.

3.1.1 Demographic

Analyses were performed for all urine specimens submitted to the Microbiology Department of the NNUH for two one-month periods (July 2014 n= 9558 urines; November 2014 n= 8991 urines). The source patients' characteristics are described in Table 12. There was no information available in the laboratory database on whether the submitted urine samples came from patients with upper *vs*. lower UTIs or complicated *vs*. uncomplicated infections. Calculations for statistics analysis are presented in details in tables in Appendix A.

Urine samples were categorized as negative when the Iris screening indicated WBC or bacteria below the standard thresholds (see Figure 15 in Section 2.1). Urines found positive by Iris, and all urines from the following locations (delivery suites, antenatal, children's wards and neonatal units, ITU and haematology wards or from patients aged < 16 years) were cultured and categorized as (i) negative (no bacterial growth), (ii) positive (heavy bacterial growth) or (iii) heavy mixed (> 2 pathogens), implying contamination rather that infection.

The number of samples submitted in July was slightly higher than in November supporting the view of the seasonality of UTI (Figure 20). Although the proportions

found negative by Iris screening, and after culture examination were similar in the two months, a Chi-square test suggested significant difference ($p < 0.05$) in the distribution of results between different categories between the July and November data.

Table 12. Characteristics of UTI patients.

Patients' characteristics	**July 2014 (n=9558)**	**November 2016 (n=8991)**
Female	6429	6090
Male	3121	2895
Unrecorded	8	6
Locations		
General Practice (GP)	6371	6201
NNUH Hospital Inpatients (H_IN)	1427	1210
NNUH Hospital Outpatients	541	282
Other Hospitals (OH)[1]	159	172
Admissions Units (AU) - Accident & Emergency (A&E) - Acute Medical Unit (AMU) - Assessment Unit (AssU) - Surgical Admissions Unit (AU)	1060 406 347 56 251	1126 338 494 70 224
Specimen types		
Bag Urine (BAG)	9	4
Catheter-Stream Urine (CSU)	658	680
Genital (GENI)	1	0
Mid-Stream Urine (MSU)	7961	7446
Supra-Pubic Aspirate (SPUB)	1	1
'Unclassified' Urine (UU)	928	860

[1] Norwich Community Hospital, Benjamin Court Hospital (Norfolk Community Health and Care NHS in Cromer), Colman Hospital (Norfolk Community Health and Care NHS Trust in Norwich), Cranmer House Residential Care in Fafenham, Dereham Hospital ((Norfolk Community Health and Care NHS), Little Plumstead Hospital in Norwich, Ogden Court Community Hospital in Wymondham, Kelling Hospital ((Norfolk Community Health and Care NHS in Cromer in Holt), Priscilla Bacon Lodge in Norwich, H.M Prison Bure in Norwich, Cromer Hospital (Norfolk Community Health and Care NHS).

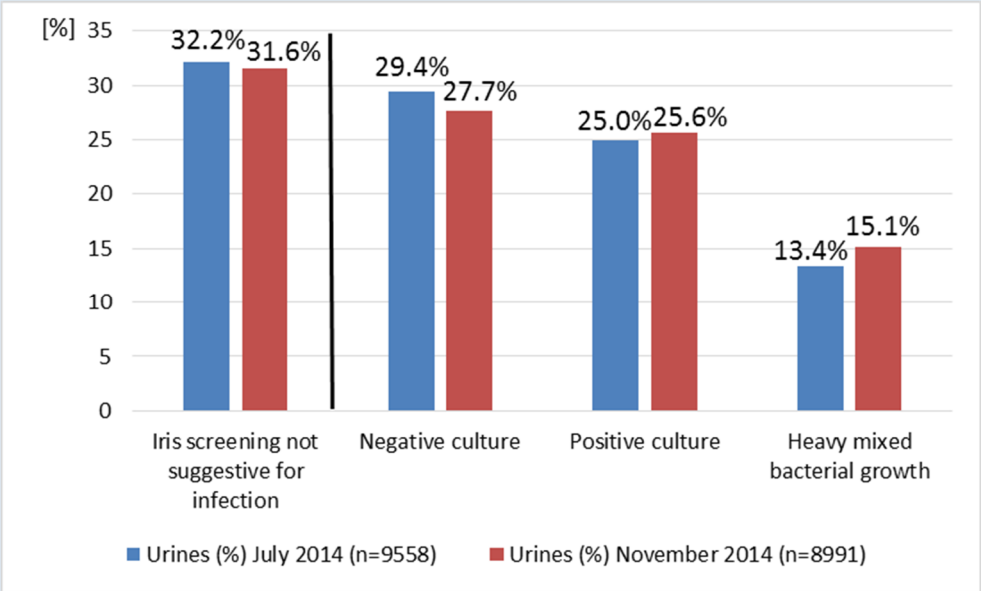


Figure 20. The proportion of urine samples submitted to the Microbiology Department of the NNUH in the two study months (July, November 2014).

In both months the largest proportion of urine examinations were performed for GP patients (> 65%), and the number of urines submitted by GPs was slightly higher in July, although the proportion was lower ($p < 0.05$). The proportion of urines from hospital inpatients and outpatients were significantly higher in July, as established by testing changes in the proportion ($p < 0.05$), while for Admission Units, the proportion was slightly higher in November than in July ($p < 0.05$), which may reflect winter pressure for admitted patients in the aged group > 85 years (Figure 21). The proportion of urines from 'other hospitals' (i.e. not the NNUH itself) was essentially the same ($p > 0.05$) in both months.

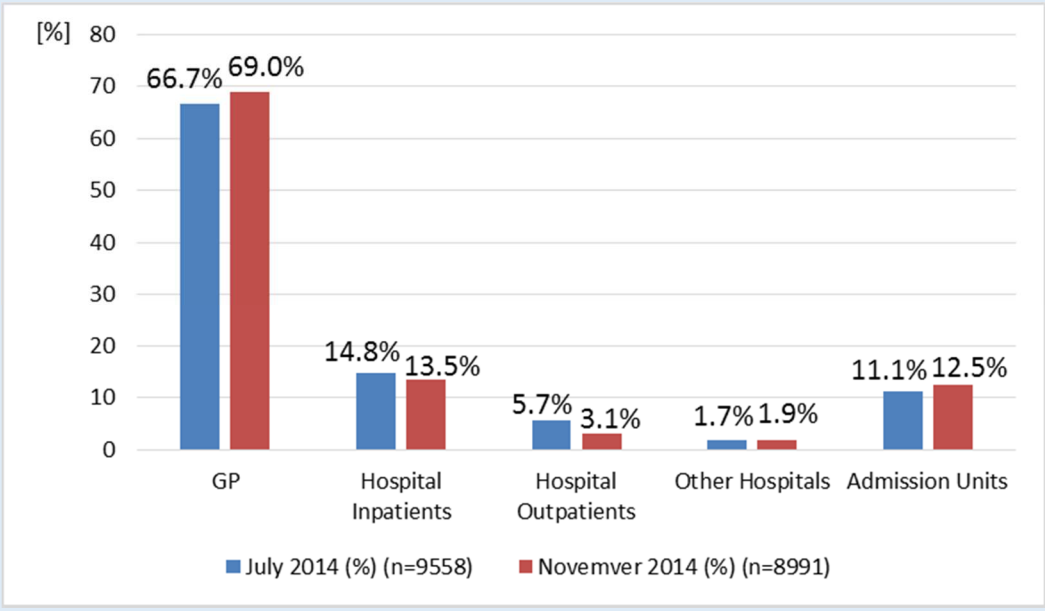


Figure 21. The proportion of urines submitted by location.

In both months women accounted for 67% of the urines. For both genders, the highest numbers of urine examinations were performed for the elderly population (66-85 year). For men the number of urine samples increased progressively with age, whereas for women there were two peaks, one in the age range 16-45 years, and the second in the age range 66-85 year (Figure 22).

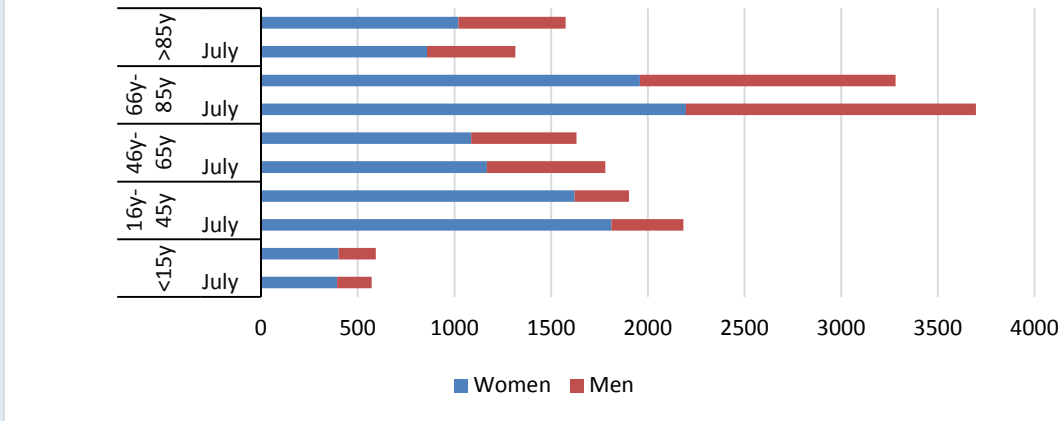


Figure 22. Total numbers of urines by gender and age group.

For each location type, the number of urines negative by Iris (blue bar Figure 23), negative by culture (red bar Figure 23), positive by culture (green bar Figure 23), and giving heavy mixed growth (violet bar Figure 23) were similar in two months. Negative urines- grouping those found negative by Iris together with those negative by culture- were the largest group, with this dominance greatest in those settings (i.e. Hospital In- and Outpatients, Admisstion Unit), where urines are cultured routinely (Figure 24, 25). GP samples had the highest proportion (28.3% for both months) of positive cultures from age 46 up to 85 in both months (Figures 26). Strategies for submitting samples for examination from GP patients remain unknown, and probably vary among individual GPs.

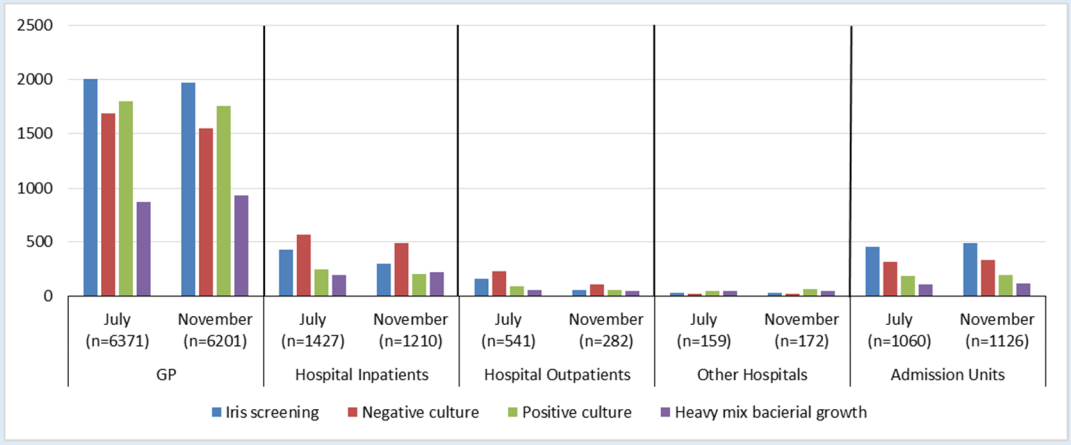


Figure 23. Total numbers of urines submitted by location.

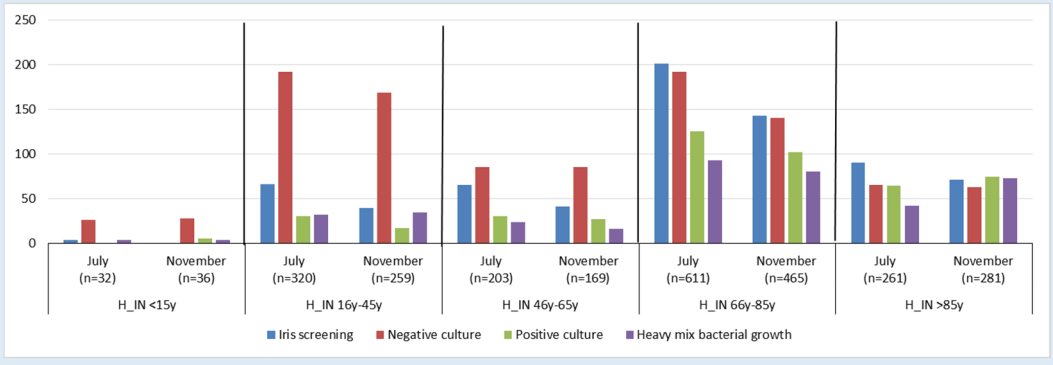


Figure 24. Total numbers of Hospital Inpatients urines submitted by age group.

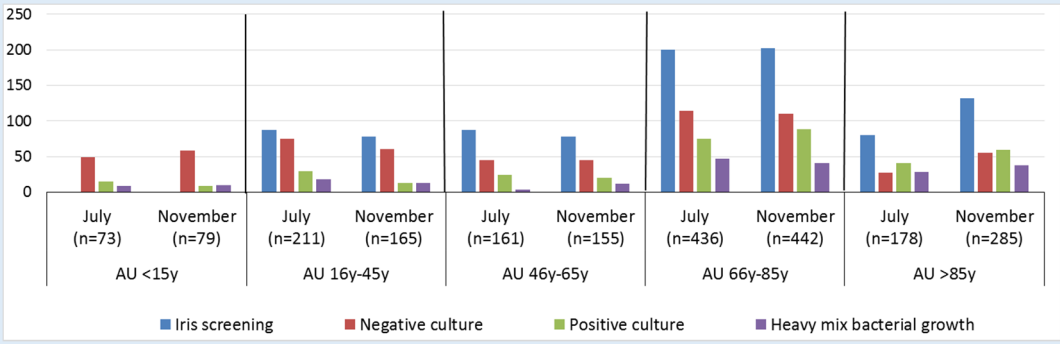


Figure 25. Total numbers of Admission Unit urines submitted by age group.

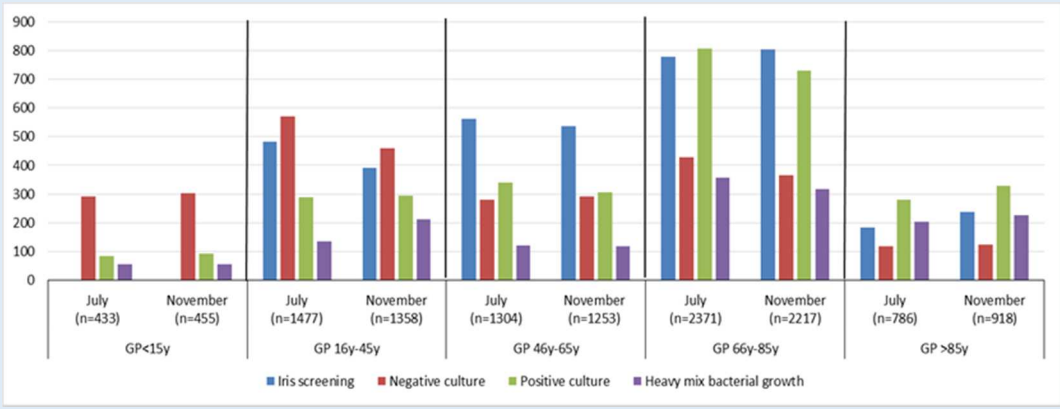
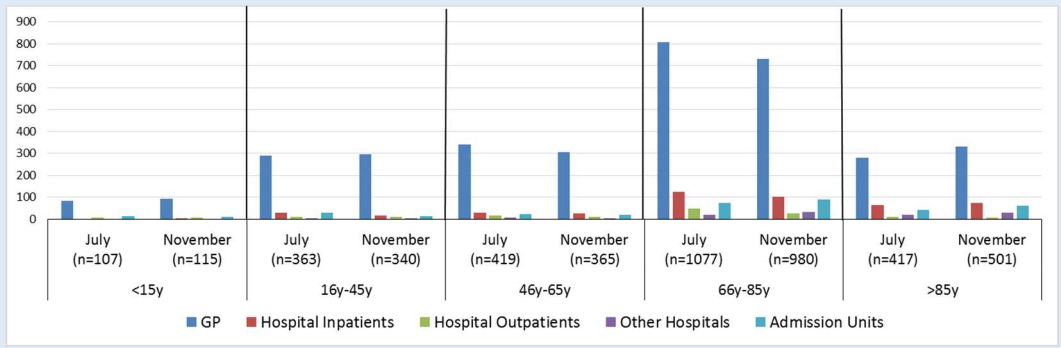


Figure 26. Total numbers of General Practice urines submitted by age group.

Overall, for both months > 67% of positive cultures in all age groups were from GP patients. The proportion of positive culture for Hospital Inpatients and Admission Unit grew slowly with the age, with the highest rates seen in 66-85 y and > 85 y age groups (Figure 27).



	<15y		16y-45y		46y-65y		66y-85y		>85y	
	July (n=107)	Nov (n=115)	July (n=363)	Nov (n=340)	July (n=419)	Nov (n=365)	July (n=419)	Nov (n=365)	July (n=419)	Nov (n=365)
GP	78.5%	81.7%	79.6%	87.1%	81.4%	83.8%	75%	75%	67.4%	69.9%
H_IN	0	4.3%	8.3%	5%	7.2%	7.4%	11.6%	10%	15.3%	14.8%
H_OUT	7.5%	6.2%	3%	3.2%	4.3%	2.5%	4.55	2.6%	2.4%	1.3%
OH	0	0	0.8%	0.9%	1.4%	0.8%	1.9%	3.3%	5%	6%
AU	14%	7.8%	8.3%	3.8%	5.7%	5.5%	7%	9.1	9.8%	12%

Figure 27. Culture positive urines by age group and location.

3.1.2 Microorganisms cultured

The most commonly-isolated pathogen was *E. coli* (68%) in both July and November. In rank order the next most frequent isolates were "Coliforms" that gave blue colonies on chromogenic agar (i.e. *Klebsiella* spp.; *Enterobacter* spp.; *Citrobacter* spp., and *Serratia* spp.) (July- 14.9%; November- 13.8%), then *Enterococcus* spp. (5.7%; 4.7%), Proteeae (4.1%; 5.2%), and *P. aeruginosa* (3.2%; 4.6%). Other Gram-positive bacteria besides i.e. enterococci accounted for small minorities- coagulase-negative staphylococci, except *S. saprophiticus,* and Group B Streptococcus each represented 1%, *S. aureus* 0.7% in both months, *S. saprophiticus* (July- 0.5%; November- 1%) and 'others' (0.1% both months) (Figure 28). Although the percentage prevalence of different pathogens was similar between the two months, differences in proportion, if we exclude 'others' reached significance ($p < 0.05$).

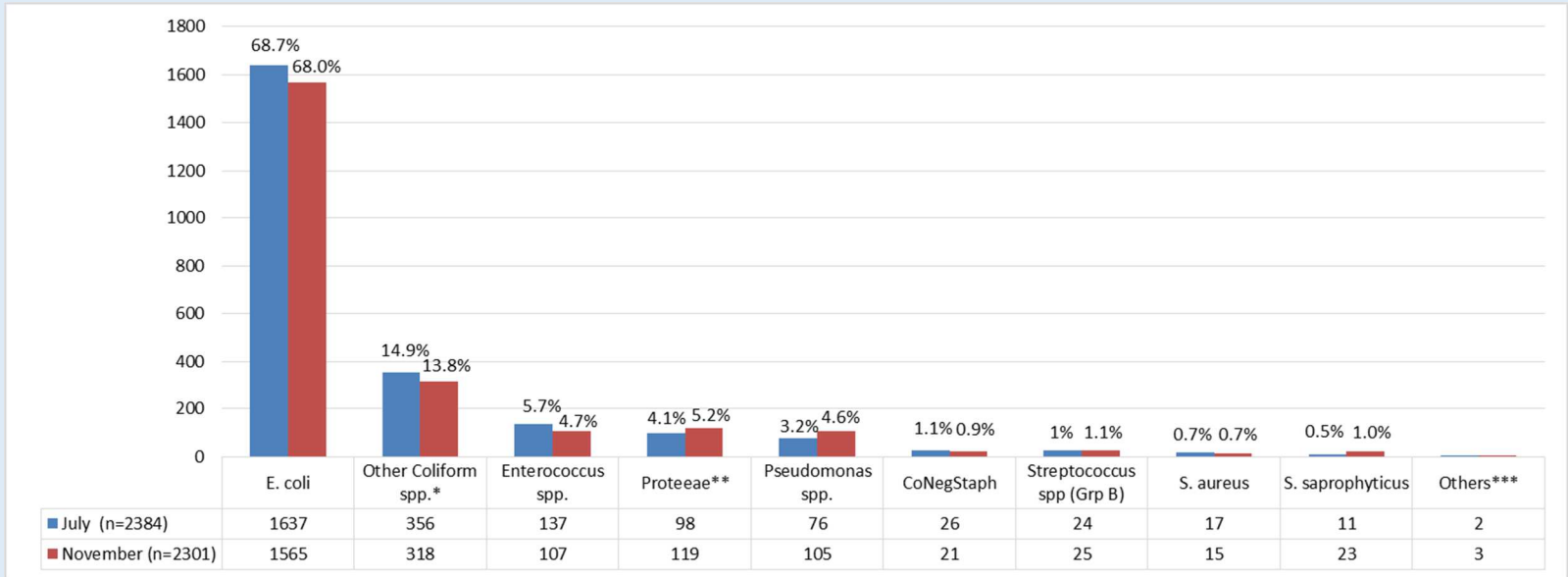


Figure 28. Proportion of different pathogens isolated from urines.

** Citrobacter* spp., *Enterobacter* spp., *Klebsiella* spp., *Serratia* spp.; ** *Morganella morganii, Providencia* spp., *Proteus* spp.; ****Aeroccocus viridans, Stenotrophomonas maltophilia*, other *Streptococcus* spp.

E.coli remained predominant in all age groups in both months. The number of *Pseudomonas* spp. and Proteeae was the highest in the elderly group (Figure 29). Among Gram-positive bacteria, *Enterococus* spp. were seen mostly in the young (≤ 15 years old) and elderly (≥ 66 years old) populations. Most *S. saprophyticus* were isolated from the 16-45 year age group in both seasons but in November, the number was twice as high compared to July (Figure 30).

Although the numbers of Gram-negative and Gram-positive uropathogens isolated were similar for female and male populations in both months, a chi-squere test showed significance differences in the pathogen distribution for women between the July and November periods ($p < 0.05$). *S. saprophyticus* was found in the female population only (Figures 31, 32).

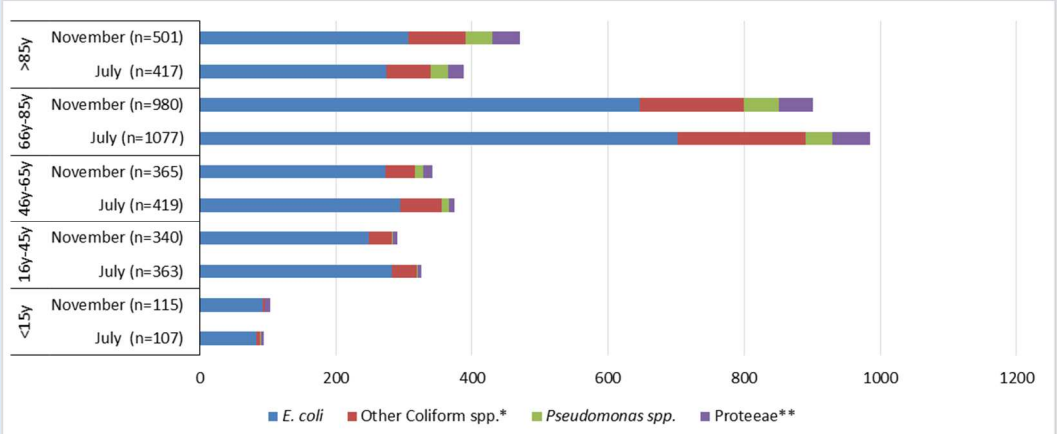


Figure 29. Gram-negative bacterial isolates by patient's age.

**Citrobacter* spp., *Enterobacter* spp., *Klebsiella* spp., *Serratia* spp.;
*** Morganella morganii, Providencia* spp., *Proteus* spp.

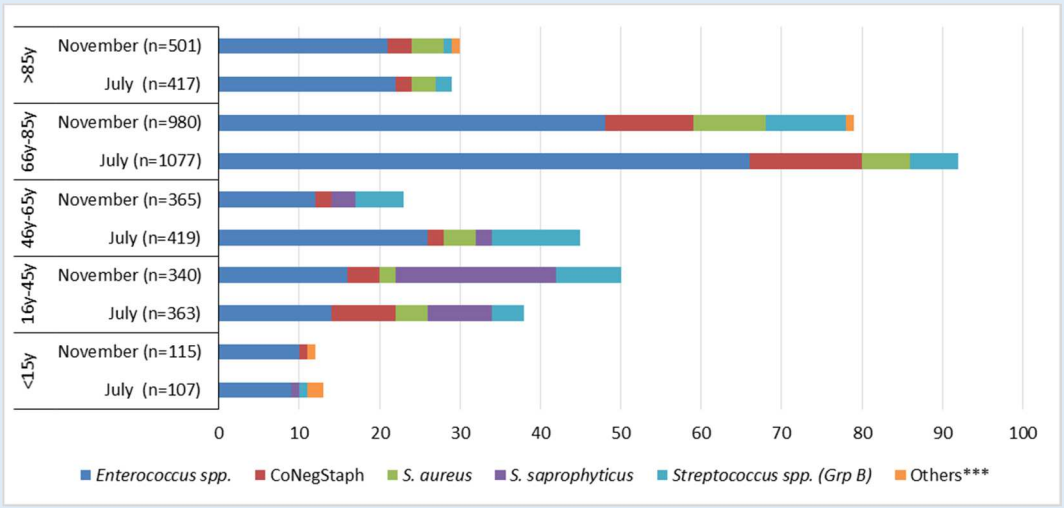


Figure 30. Gram-positive bacterial isolates by patient's age.

*** *Aeroccocus viridans* and other *Streptococcus* spp.

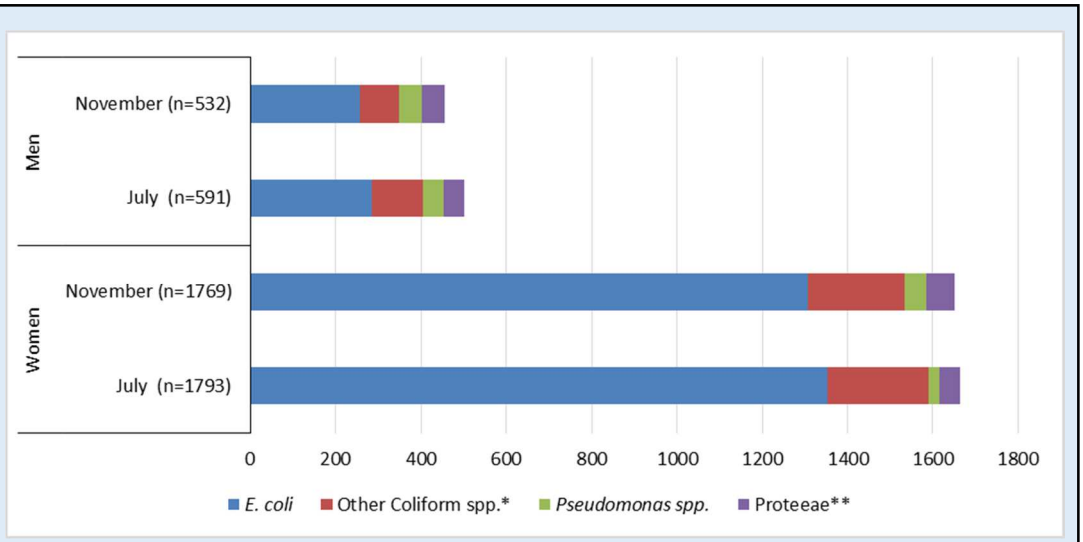


Figure 31. Gram-negative bacterial isolates by gender.

* *Citrobacter* spp., *Enterobacter* spp., *Klebsiella* spp., *Serratia* spp.;
** *Morganella morganii, Providencia* spp., *Proteus* spp.

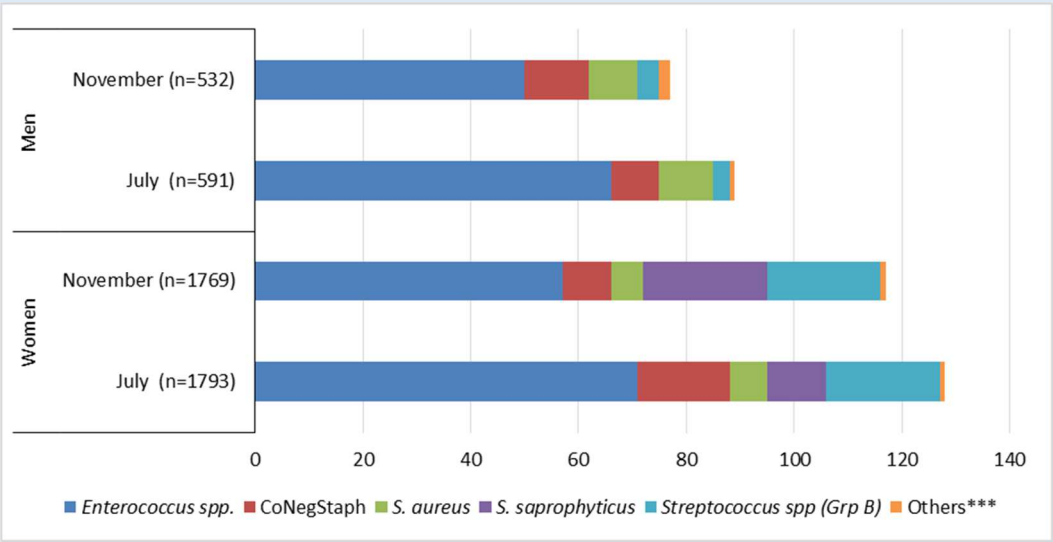


Figure 32. Gram-positive bacterial isolates by gender.

*** *Aeroccocus viridans* and other *Streptococcus* spp.

3.1.3 Urine sample types

In both months, the most common specimen type collected from the patients were mid-stream urines (MSU) (83%). Bag urines (BAG) and catheter-stream urines (CSU) were small minorities (collectively ~7%) (Table 12). The remaining 10% were mostly 'unclassified' urines (UU). Unsurprisingly, the percentage of insignificant heavy mixed bacterial growth in BAG/CAT samples was twice as high as among MSU samples (Figure 33). Although *E. coli* predominant in all samples type in both months, *Pseudomonas* spp. and other Coliform species represented much higher proportion in the BAG and CAT specimens than in MSU (Figure 34).

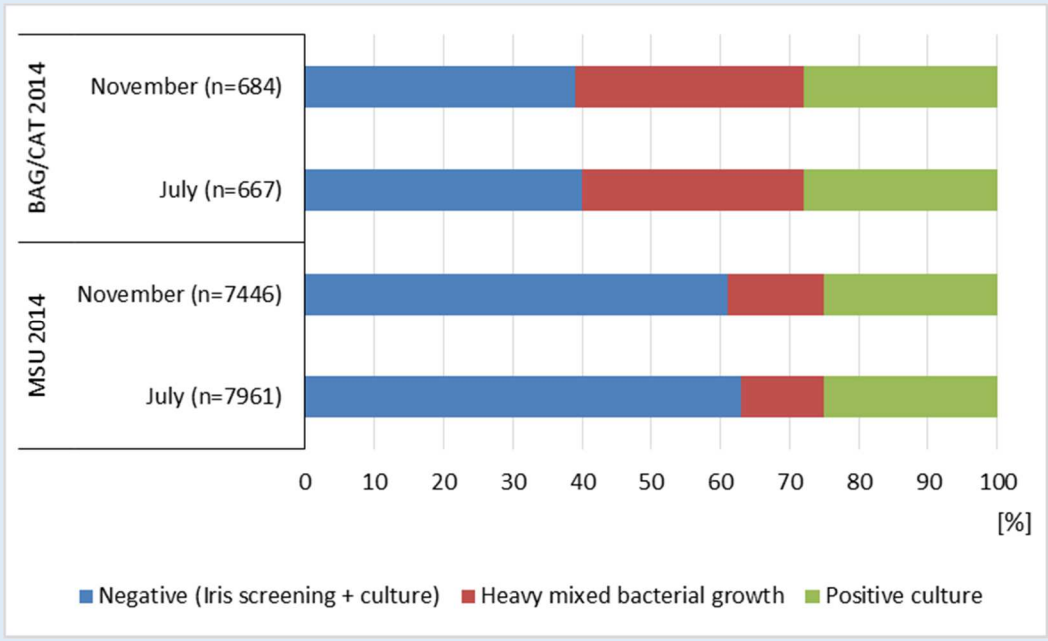


Figure 33. Microbiology results by sample type.

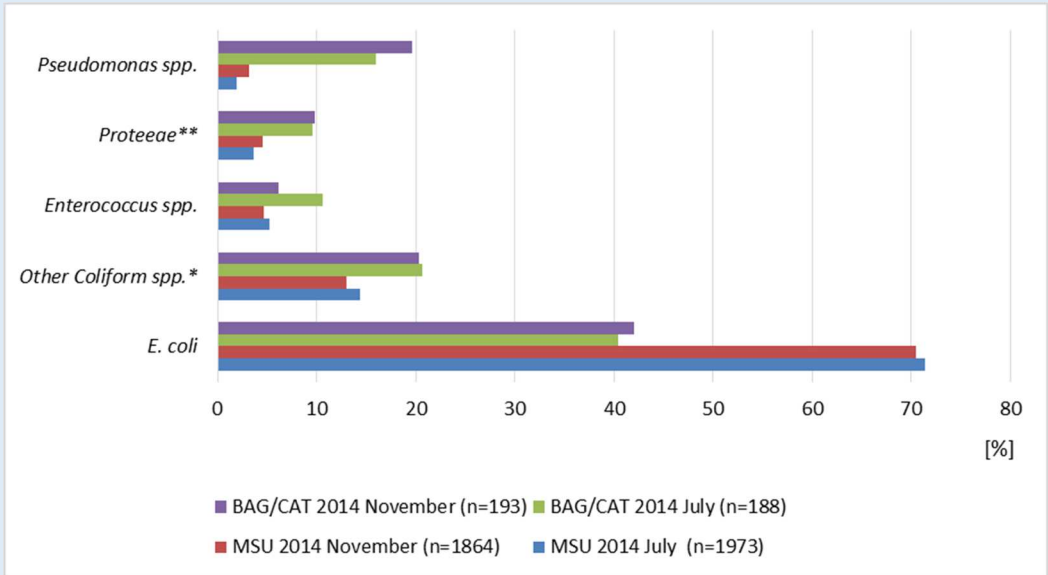


Figure 34. Percentage prevalence of different organisms by sample type.

* *Citrobacter* spp., *Enterobacter* spp., *Klebsiella* spp., *Serratia* spp.

** *Morganella morganii, Providencia* spp., *Proteus* spp.

3.1.4 Resistance rates among urine isolates

In both months, the resistance rates among *E. coli* were highest for amoxicillin and trimethoprim for all locations. In July the resistance rates for amoxicillin exceeded 50% for GP patients, Hospital Inpatients, Hospital Outpatients (50.6%, 54.5%, 56.5%) respectively, while for Admission Unit and 'other hospitals' the rates were 45.8% and 45.8%, respectively. In November the amoxicillin resistance rate for GP urines was 49% compared with 61.3% for Hospital Inpatients, 52.9% for Admission Unit and 57.1% for 'other hospitals', but only 33.3% for Hospital Outpatients.

The resistance rate for *E. coli* to trimethoprim exceeded 30% in both July and November for GP (32.8%, 33.8%), Hospital Inpatients (44.9%, 34.7) and the Admission Unit (33.9%, 36.4%). For Hospital Outpatients isolates the trimethoprim resistance rate was two-fold lower in November than in July (17.9% *vs.* 35.5%) whilst for 'other hospitals' the trimethoprim resistant rate was higher in November than in July (40.5% *vs.* 25%). These show sharp fluctuation, probably arising by chance of initial empirical policy, which may vary in efficiently across short periods.

Resistance rates for 3rd generation cephalosporins (cefpodoxime, ceftriaxone) were in a range 2-13% for all locations in both months (Figure 35).

Although among *E. coli* the proportions of isolates resistant to amoxicillin and trimethoprim varied in different locations between the two months the differences in the proportion were not statistically significant ($p > 0.05$), except for amoxicillin in hospital outpatients ($p < 0.05$).

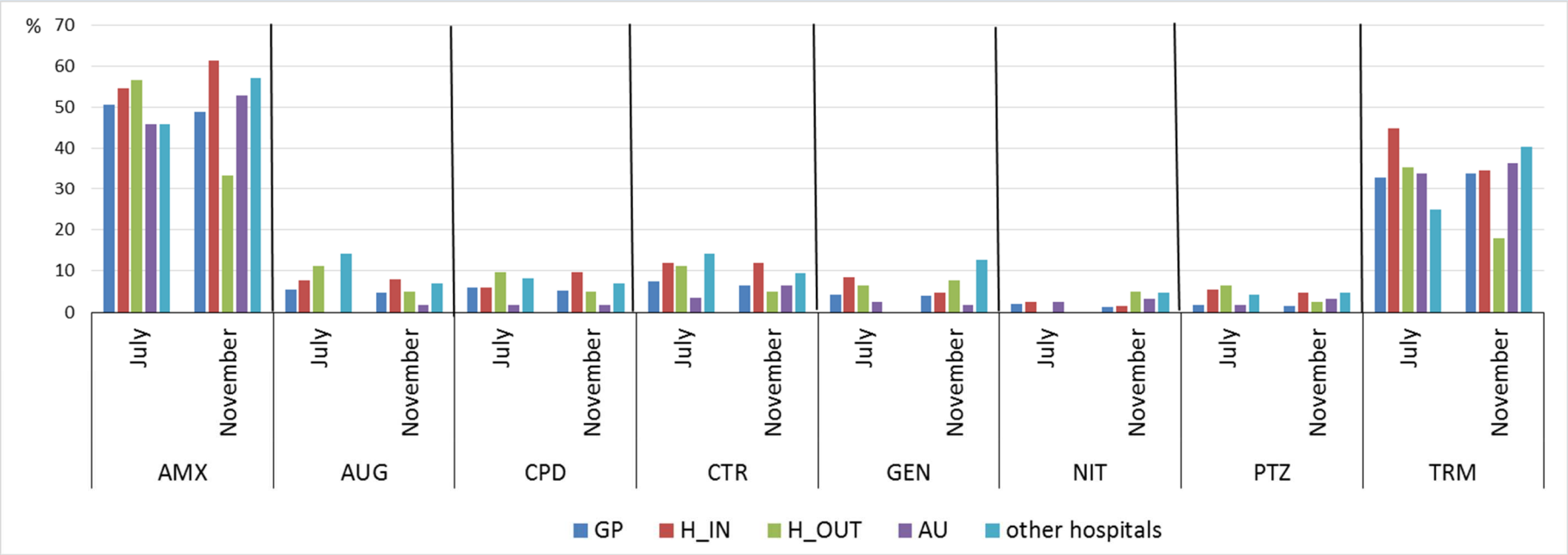


Figure 35. Percentage antibiotic resistance rates among *E. coli* by location.

July (*E. coli* n=1637): GP (n=1266); H_IN (n=167); H_OUT (n=62); AU (n=118); other hospitals (n=24)

November (*E. coli* n=1565): GP (n=1239); H_IN (n=124); H_OUT (n=39); AU (n=121); others hospital (n=42) Legend and content of discs used for testing: AMX- amoxicillin (10 µg); AUG- co-amoxiclav (20 + 10 µg); CPD- cefpodoxime (10 µg); CTR- ceftriaxone (30 µg); GEN- gentamicin (10 µg); NIT- nitrofurantoin (200 µg), PTZ- piperacillin/tazobactam (75+10 µg), and TRM- trimethoprim (2.5 µg).

The trimethoprim resistance rate for other coliform species (*Citrobacter* spp., *Enterobacter* spp., *Klebsiella* spp., and *Serratia* spp.) was < 30% for both months, except for Admission Unit in July (34.6%) although the number of patients were small. Larger proportion of these species were resistant to nitrofurantoin compared to *E. coli,* probably reflecting inherent resistance in some of these species. In both months the resistance rates for 3rd generation cephalosporins (cefpodoxime, ceftriaxone) in the other coliform groups was around 10-15%, except for Hospital Inpatients in July, where the rate exceeded 25% (Figure 36).

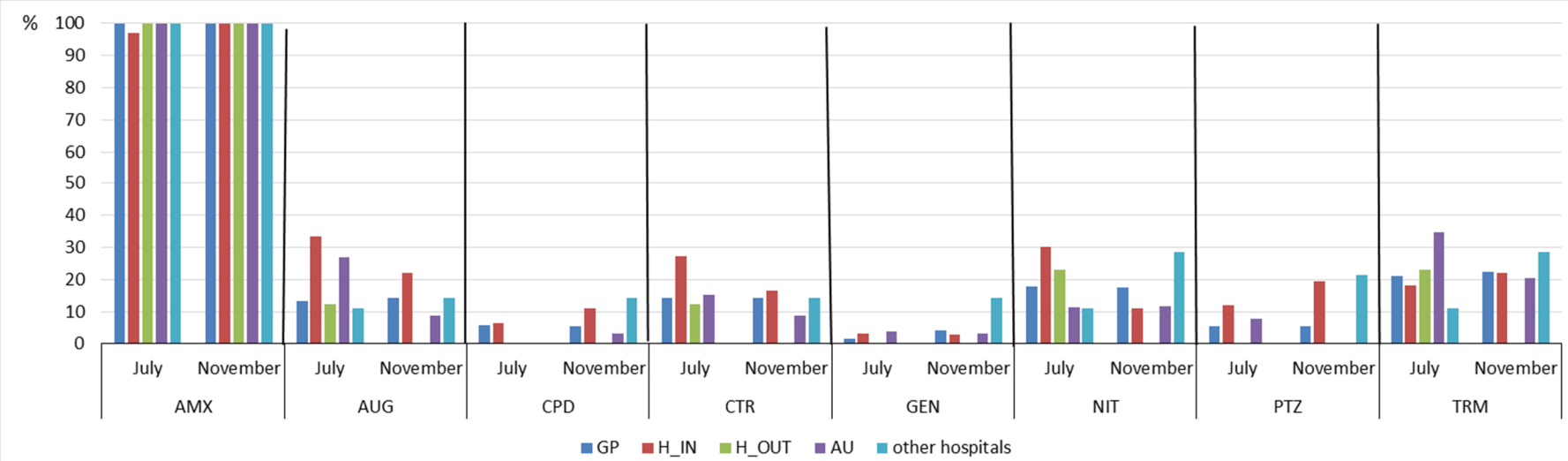


Figure 36. Percentage antibiotic resistance for other coliform species*, excluding *E. coli,* by location.

**Citrobacter* spp., *Enterobacter* spp., *Klebsiella* spp., and *Serratia* spp.

July (Coliform species n=356): GP (n=272); H_IN (n=33); H_OUT (n=16); AU (n=26); other hospitals (n=9)

November (Coliform species n=318): GP (n=231); H_IN (n=36); H_OUT (n=3); AU (n=34); other hospitals (n=14)

Abbreviations as for Figure 35.

In both months, the majority of *Pseudomonas* spp. isolates were susceptible to gentamicin and piperacillin/tazobactam. For Proteeae the data showed 20% resistance to amoxicillin, and approximately 40% resistance to trimethoprim for both months (Table 13). This may reflect natural resistance to amoxicillin in *Morganella morganii, Proteus vulgaris* or *Providencia* spp. or acquired resistance in *Proteus mirabilis*. Antibiotic resistance rates for other isolated bacterial species are presented in Table 13. All *Enterococcus* spp., *Streptococcus* gr B and *S. saprophyticus* isolates were susceptible for amoxicillin, co-amoxiclav and nitrofurantoin in July whereas resistant isolates were seen in the November. The high overall amoxicillin susceptibility rate suggests that *E. faecalis* predominated among *Enterococcus* spp.; the small but raised proportion with resistance to amoxicillin and nitrofurantoin suggests a small minority of *E. faecium* isolated in November.

Table 13. Numbers of Proteeae and non-Enterobacteriaceae bacterial species isolated and their resistance to antibiotics.

Pathogens	Month (total)	AMX	AUG	CPD	CRO	GEN	NIT	PTZ	TMP
Enterococcus spp.	July (n=137)	0	0	-	-	-	1	-	-
	Nov (n=107)	10	9	-	-	-	5	-	-
Proteeae	July (n=98)	24	7	2	9	4	97[1]	0	35
	Nov (n=119)	27	7	1	7	1	118[1]	0	40
Pseudomonas spp.	July (n=76)	-	-	-	-	1	-	1	-
	Nov (n=105)	-	-	-	-	1	-	0	-
CoNegStaph (excluding *S. saprophyticus*)	July (n=26)	14	5	-	-	-	-	-	-
	Nov (n=21)	16	7	-	-	-	-	-	-
*Strep*tococcus gr B	July (n=24)	0	0	-	-	-	0	-	-
	Nov (n=25)	0	0	-	-	-	1	-	-
S. aureus	July (n=17)	10	1	-	-	-	-	-	-
	Nov (n=15)	8	2	-	-	-	-	-	-
S. saprophyticus	July (n=11)	0	0	-	-	-	0	-	-
	Nov (n=23)	12	8	-	-	-	0	-	-

[1] Proteeae are inherently resistant to nitrofurantoin.

Abbreviations as for Figure 35.

3.2 Matrix-Assisted Laser-Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS)

AIMS:

- To evaluate mass spectrometry for bacterial identification directly from clinical urine samples, without culture.
- To detect hydrolysis of cephalosporins by bacteria harvested directly from urines, again without culture.

3.2.1 Identification of bacteria directly from urine

3.2.1.1 Optimisation of the sample preparation procedure for pathogen identification by MALDI-TOF

The main objective of this section was to optimize the sample preparation for rapid identification of pathogens directly from urine samples. To achieve this, several factors were evaluated including: (i) the duration and speed of the first centrifugation step used to remove human cells, (ii) the instrument detection limit, (iii) the effects of boric acid, antibiotics, chemical reagents which may be present in, or added to urines being processed, (iv) the presence of two bacterial species in the urine.

Retention of bacterial cells during removal of human cells

Two millilitre volumes of urine spiked with bacteria with a count of 3.2 x 10^8 cfu/mL (*E. coli),* 4.2 x 10^8 cfu/mL (*K. pneumoniae),* 2.9 x 10^8 cfu/mL (*P. mirabilis),* 3.5 x 10^8 cfu/mL (*P. aeruginosa*), 1.14 x 10^8 cfu/mL (*E. faecalis*) and 4.2 x 10^8 cfu/mL (*S. aureus*) were analysed to optimise the duration and speed of the first centrifugation step, which aimed to pellet human cells but not bacteria. The following combinations of parameters were tested, all of which are accepted as being able to pellet human cells (Ferreira *et al.*, 2010).

a) 2000 rpm-30 sec; b) 2000 rpm-2 min; c) 2000 rpm-5 min; d) 3000 rpm-2 min; e) 3000 rpm-5 min; f) 4000 rpm-2 min; g) 4000 rpm-5 min; h) 5000 rpm-2 min; i) 5000 rm-5 min.

The number of bacteria remaining in the supernatant decreased progressively as the centrifugation speed and duration increased, leading to >80% loss of bacteria. Optimal conditions were assessed to be 2000 rpm (300 *g*) for 2 min for both Gram-negative and Gram-positive bacteria. The results are presented in Figure 37.

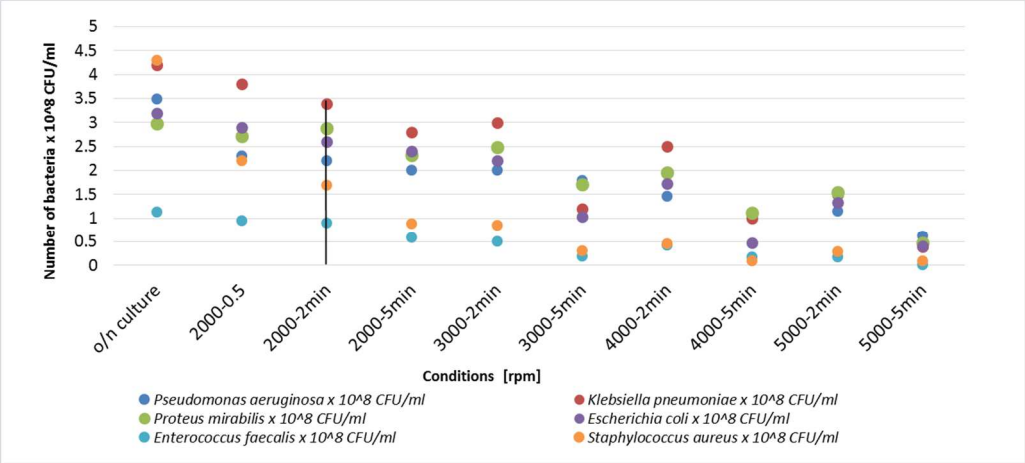


Figure 37. Number of Gram-negative and Gram-positive bacteria remaining in the supernatant after different first centrifugation steps.

Detection limit

Detection limits were determined using ten-fold dilutions of urines spiked with bacterial counts 1.53 x 10^9 cfu/mL (*E. coli),* 3.1 x 10^9 cfu/mL (*K. pneumoniae),* 5.44 x 10^9 cfu/mL (*P. mirabilis),* 1.88 x 10^9 cfu/mL (*P. aeruginosa*), 6.5 x 10^9 cfu/mL (*E. faecalis*) and 0.35 x 10^8 cfu/mL (*S. aureus*). Ordinary a MALDI-TOF score of >2.0 is required for species identification and a score of >1.7 for genus identification. To increase the number of species and genus identification directly on urine lower scores (>1.200 for genus) were accepted. Results are shown in Table 14. MALDI-TOF scores >2.00 were reliable obtained with bacterial counts >10^7 cfu/mL, but if scores >1.200 were accepted bacteria was reliable identified with density in the 10^5-10^6 range.

Table 14. Detection limit of the MALDI-TOF for uropathogens.

Pathogen	Colony count by overnight culture (o/n)	MALDI-TOF scores					
		Dilutions of culture					
		o/n	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
E. coli	1.53 x 10^9 cfu/mL	2.350	2.498	2.265	1.416	1.425	NPF
K. pneumonia	3.10 x 10^9 cfu/mL	2.427	2.517	2.423	1.671	1.410	NPF
P. mirabilis	5.44 x 10^9 cfu/mL	2.344	2.400	2.267	1.310	1.527	NPF
P. aeruginosa	1.88 x 10^9 cfu/mL	2.484	2.448	2.218	1.515	NPF	NPF
E. faecalis	6.50 x 10^9 cfu/mL	2.512	2.445	2.156	1.248	1.308	NPF
S. aureus	0.35 x 10^8 cfu/mL	2.427	2.336	2.140	1.306	1.341	NPF

Legend: NPF- no peak found. Based on Brüker's criteria a score >2.00 indicates species identification; 1.700-1.999 indicates genus level; <1.699 unreliable identification. Nevertheless experience indicated that results were reproductible and reliable obtained down to a score of 1.200.

Effect of boric acid on MALDI-TOF score

The effect of boric acid (1-2%) in the container was tested using urine spiked with bacterial counts 3.2 x 10^8 cfu/mL (*E. coli),* 7.0 x 10^8 cfu/mL (*K. pneumoniae),* 4.6 x 10^8 cfu/mL (*P. mirabilis),* 3.5 x 10^8 cfu/mL (*P. aeruginosa*), 1.14 x 10^8 cfu/mL (*E. faecalis*) and 4.3 x 10^8 cfu/mL (*S. aureus*). MALDI-TOF analysis was performed during up to 7 days storage in the fridge (4-8°C) of urine in the containers with and without boric acid using both the MALDI-TOF extraction procedure with formic acid/acetonitrile and by direct spotting of the washed bacterial pellet onto the target plate (see Figure 16 Section 2.2).

The presence of boric acid in the container did not interfere with MALDI-TOF analysis and did not affect the MALDI-TOF scores for common UTI pathogens. For Gram-negative bacteria, a score > 2.00, with no significant drop-off over time, was achieved by both the direct spot and by extraction over all seven days of analysis (Figures 38, 39, 40, 41). Likewise for Gram-positive bacteria (Figures 42, 43), the scores did not decline over time regardless of the presence of boric acid; however the

extraction step was critical to provide genus identification with a score > 2.000, especially when the analysis was delayed beyond 24 hours.

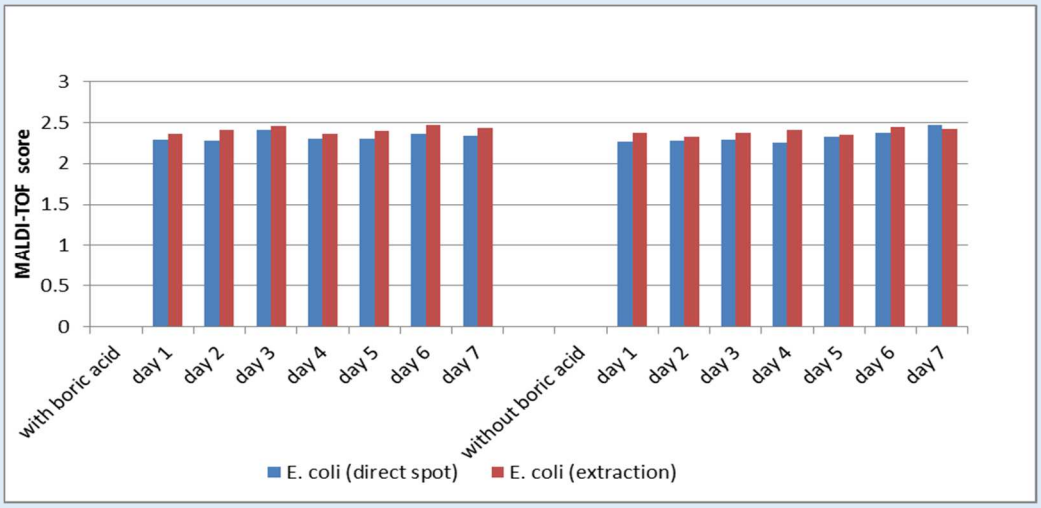


Figure 38. The effect of boric acid on MALDI-TOF scores for *E. coli* in urine.

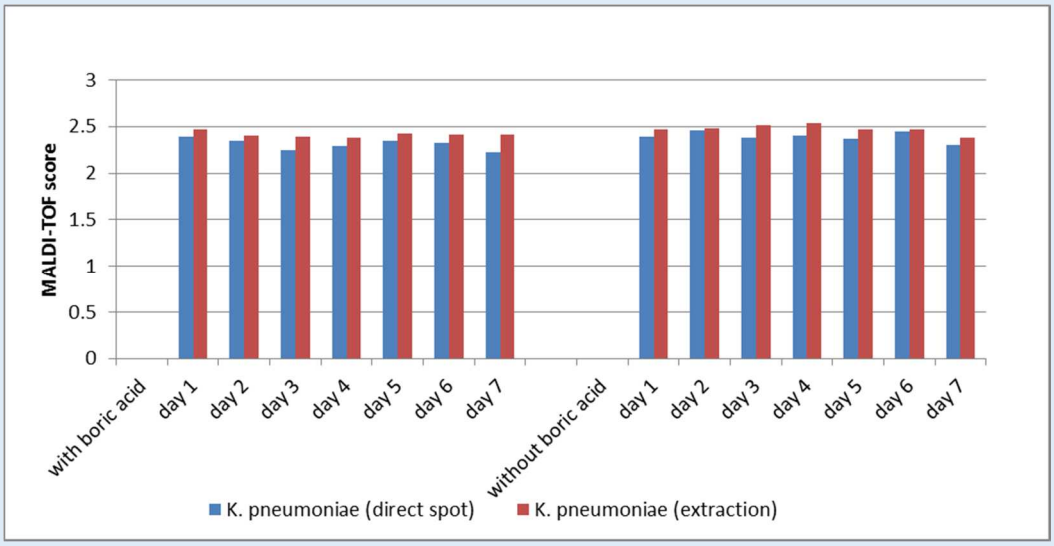


Figure 39. The effect of boric acid on MALDI-TOF scores for *K. pneumoniae* in urine.

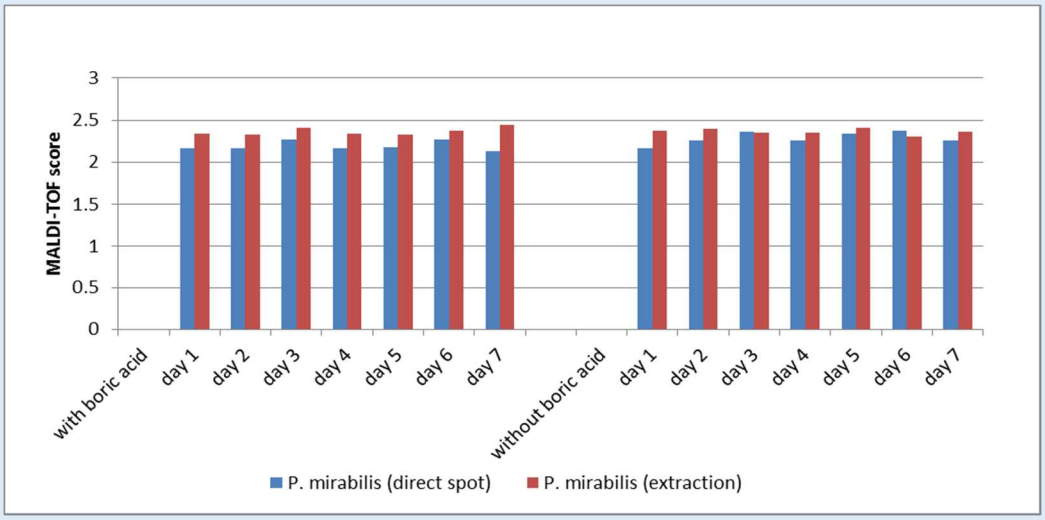


Figure 40. The effect of boric acid on MALDI-TOF scores for *P. mirabilis* in urine.

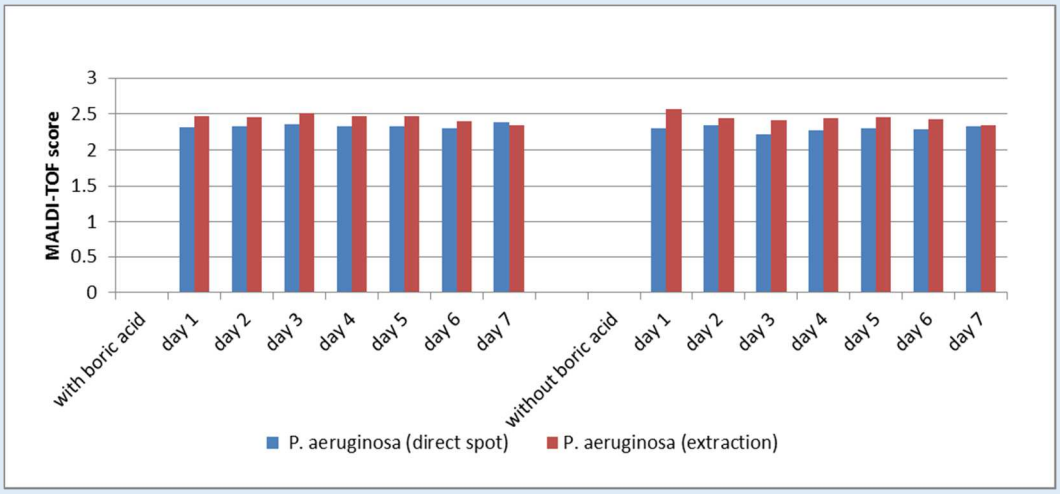


Figure 41. The effect of boric acid on MALDI-TOF scores for *P. aeruginosa* in urine.

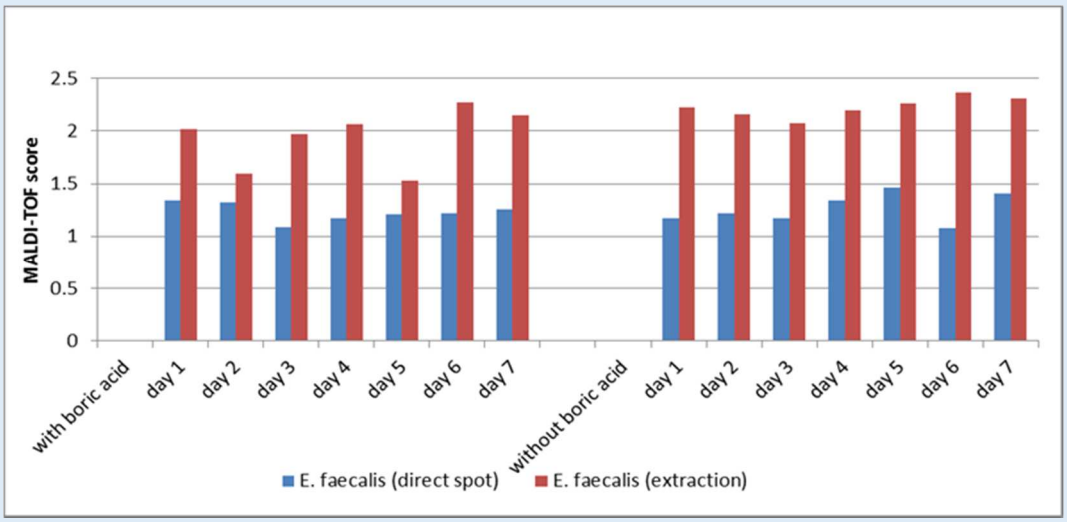


Figure 42. The effect of boric acid on MALDI-TOF scores for *E. faecalis* in urine.

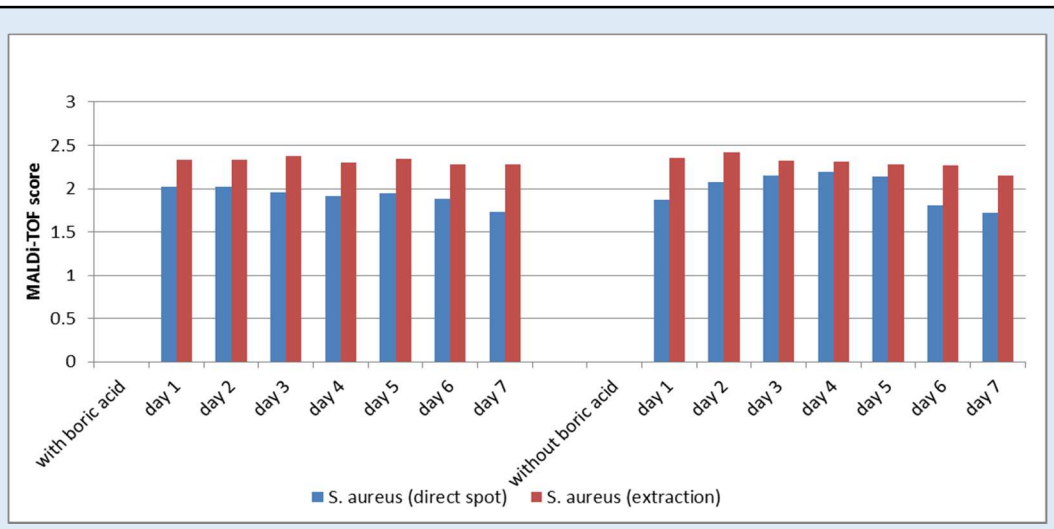


Figure 43. The effect of boric acid on MALDI-TOF scores for *S. aureus* in urine.

Effect of antibiotics on MALDI-TOF score

The effect of antibiotics on MALDI-TOF scores was investigated using urine in boric acid containers spiked with bacterial densities as follows 2.9 x 10^8 cfu/mL for *E. coli,* 4.5 x 10^8 cfu/mL for *P. aeruginosa,* 3.4 x 10^8 cfu/mL for *E. faecalis,* and 2.1 x 10^8 cfu/mL for *S. aureus*. The presence of boric acid had already been shown not to affect MADLI-TOF scores over time.

The presence of antibiotics in urine (ciprofloxacin-100 mg/L, trimethoprim-200 mg/L and trimethoprim/sulfamethoxazole- 100 mg/L+500 mg/L) did not interfere with MALDI-TOF analysis, and did not affect the MALDI-TOF score over time. For *E. coli* and *P. aeruginosa*, MALDI-TOF scores were >2.00 for both the direct spot and extraction methods during up to 6 days of storage in the fridge (4-8°C) (Figures 44, 45) regardless of the presence of these antibiotics. For *E. faecalis* and *S. aureus* scores also remained unchanged over time; again however the extraction step was necessary to ensure scores >2.00 (Figures 46, 47), especially when the analysis was delayed beyond 24 hours.

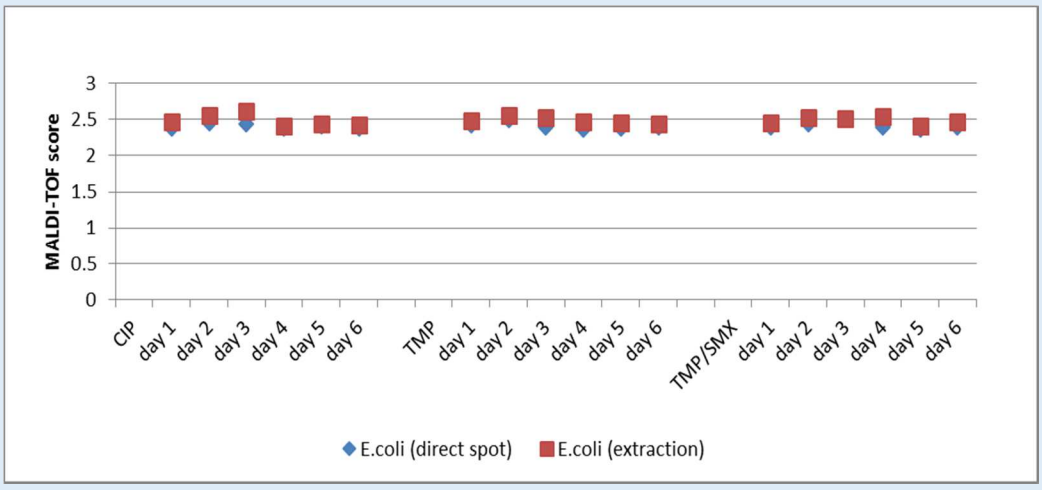


Figure 44. The effect of antibiotics on MALDI-TOF scores for *E. coli* in urine.

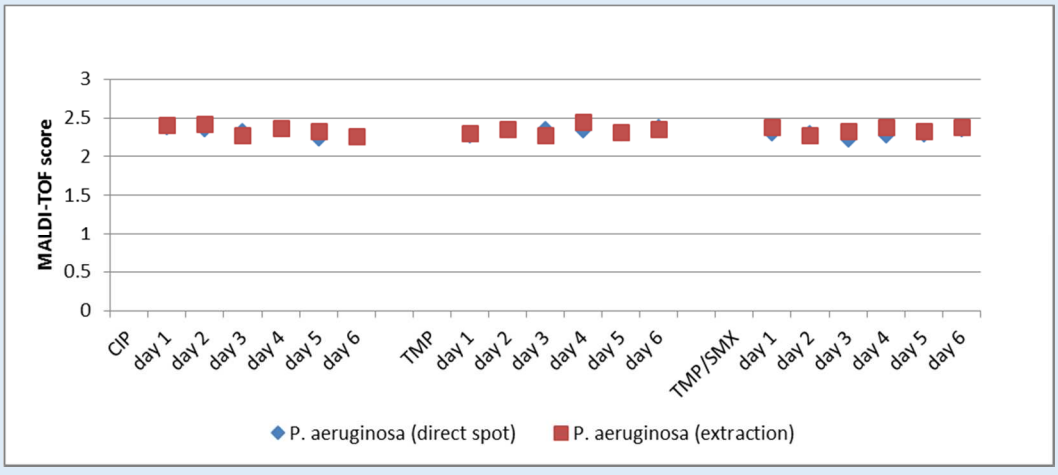


Figure 45. The effect of antibiotics on MALDI-TOF scores for *P. aeruginosa* in urine.

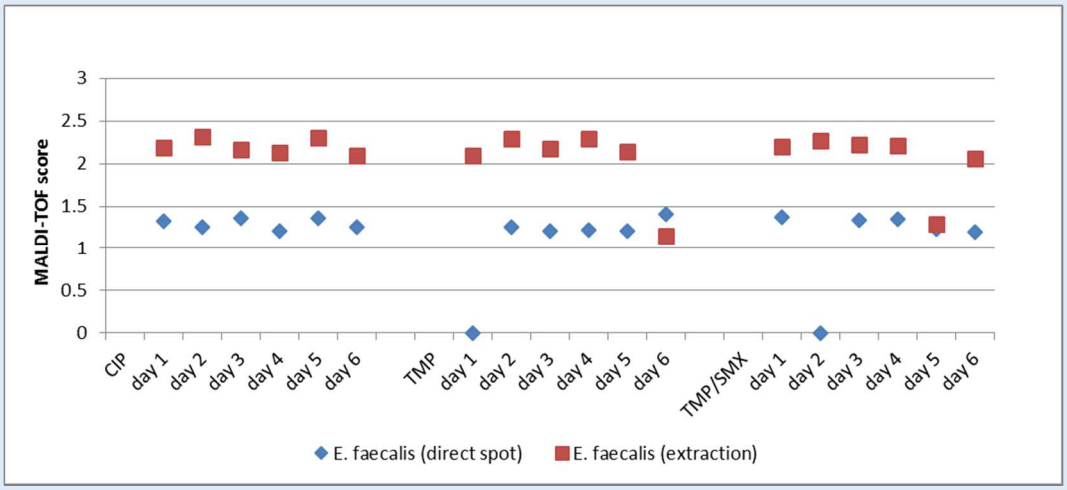


Figure 46. The effect of antibiotics on MALDI-TOF scores for *E. faecalis* in urine. No peaks were found for the TMP and TMP/SMX on day 1 and day 2, respectively by the direct spot indicating processing failures.

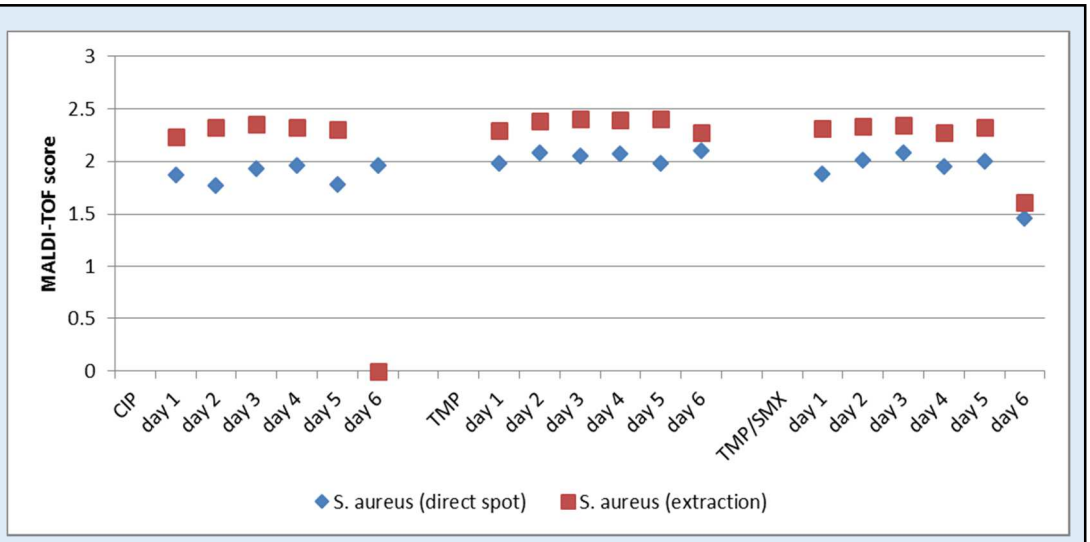


Figure 47. The effect of antibiotics on MALDI-TOF scores for *S. aureus* in urine**.** There was a test failure with the extraction method on the day 6 with ciprofloxacin.

Presence of two microorganisms in urine specimens

Mixtures of pairs of organisms were prepared by adding different ratios of urines spiked with bacterial densities: 0.26 x 10^9 cfu/mL; 0.33 x 10^9 cfu/mL; 0.5 x 10^8 cfu/mL; 0.15 x 10^9 cfu/mL of *E. coli*; *P. aeruginosa*; *E. faecalis* and *S. aureus,* respectively. Two-millilitre volumes of each mixture were analysed on the MALDI-TOF using the protocol presented in Figure 16 (see section 2.2). Result for urine specimens containing two microorganisms are shown in Table 15. When two specimens were present in similar proportion (e.g. 0.8 : 1 for an *E. coli* and *P. aeruginosa* mixture or 1 : 0.38 for an *E. coli* and *E. faecalis* mixture) both species were simultaneously recognized; at more extreme ratios (e.g 1:8) only the predominant bacterial was detected.

Table 15. MALDI-TOF analysis with mixed bacteria populations.

	Ratio (volume)	Ratio count	Species detected 1	Species detected 2
E. coli: P.aeruginosa	10:1	7.9 : 1	*E.coli*	-
	4:1	3.2 : 1	*E. coli*	-
	3:1	2.4 : 1	*E. coli*	-
	2:1	1.6 : 1	*E. coli*	*P. aeruginosa*
	1:1	0.8 : 1	*E. coli*	*P. aeruginosa*
	1:2	1 :2.5	*E. coli*	*P. aeruginosa*
	1:3	1 : 3.8	*E.coli*	*P. aeruginosa*
	1:4	1 : 5.1	*E. coli*	*P. aeruginosa*
	1:10	1 :12.7	-	*P. aeruginosa*
E. coli: E. faecalis	10:1	52 : 1	*E. coli*	-
	4:1	20.8 : 1	*E. coli*	-
	3:1	15.6 : 1	*E. coli*	-
	2:1	10.4 : 1	*E. coli*	-
	1:1	5.2 : 1	*E. coli*	*E. faecalis*
	1:2	1 : 0.38	*E. coli*	*E. faecalis*
	1:3	1 : 0.6	*E.coli*	*E. faecalis*
	1:4	1 : 0.8	*E. coli*	*E. faecalis*
	1:10	1 : 1.9	*E. coli*	*E. faecalis*
E. faecalis: S. aureus	10:1	3.3 : 1	*E. faecalis*	*S. aureus*
	4:1	1.3 : 1	*E. faecalis*	*S. aureus*
	3:1	1 : 1	*E. faecalis*	*S. aureus*
	2:1	0.67 : 1	*E. faecalis*	*S. aureus*
	1:1	0.33 : 1	*E. faecalis*	*S. aureus*
	1:2	1 : 6	*E. faecalis*	*S. aureus*
	1:3	1 : 9	-	*S. aureus*
	1:4	1 : 12	-	*S. aureus*
	1:10	1 : 30	-	*S. aureus*

Effect of chemical reagents on MALDI-TOF

None of the chemical reagents added aiming to remove human cells (listed in the Table 5 Section 2.2.1) improved the results of MALDI-TOF analysis, with the exception of 1% SDS. Addition of 1% SDS increased the scores on MALDI-TOF for Gram-negative and Gram-positive bacteria, as presented in Figure 48. The use of lysozyme (1 mg/mL) together with 1% SDS further improved identification scores (> 1.5) for Gram-positive bacteria as shown in Figure 49.

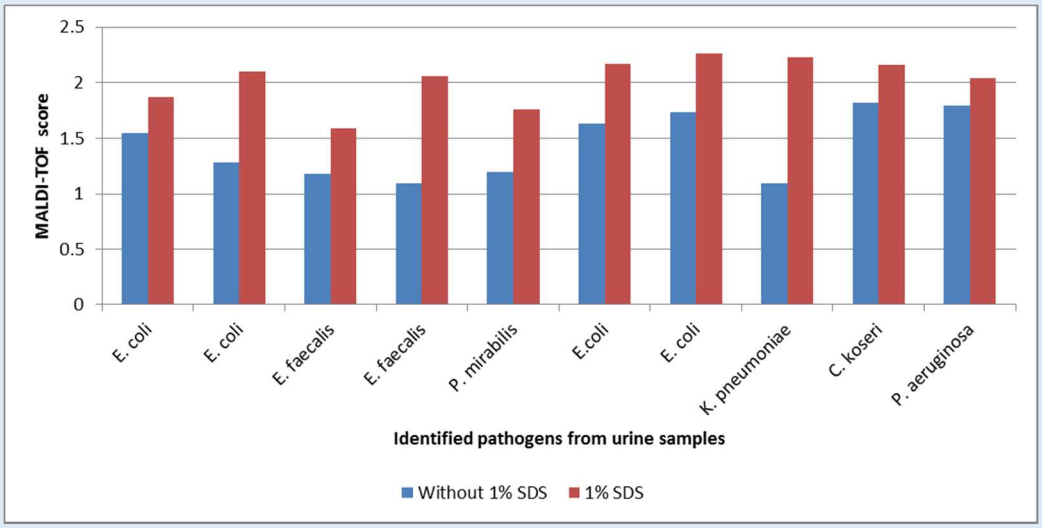


Figure 48. The effect of SDS (1%) on MALDI-TOF scores using the extraction method for Gram-negative and Gram-positive bacteria directly on urine specimens.

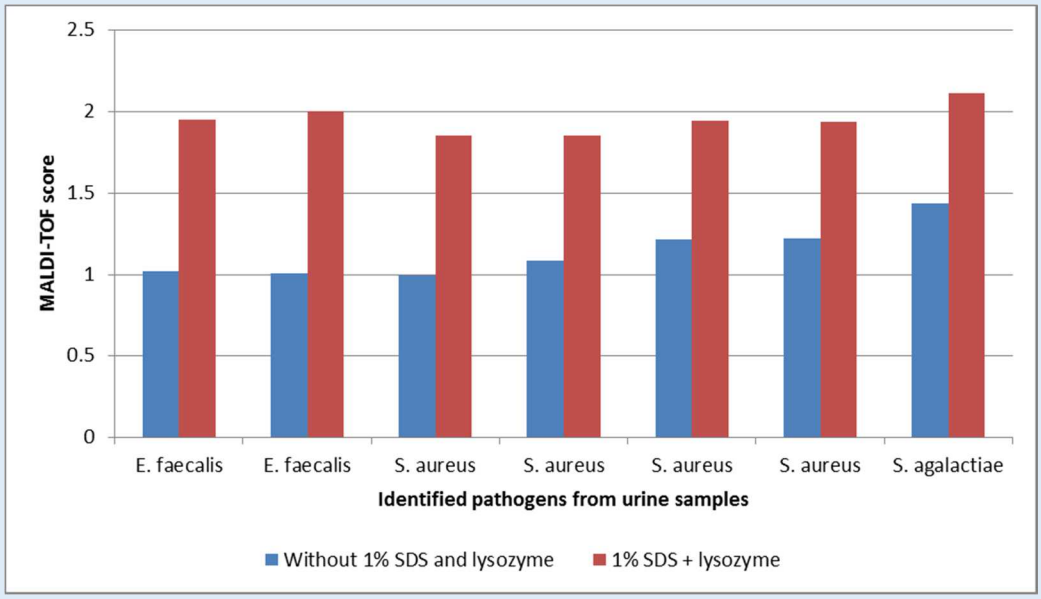


Figure 49. The effect of SDS (1%) and lysozyme on MALDI-TOF scores using the extraction method for Gram-positive bacteria directly on urine specimens.

3.2.2 In-use performance of rapid identification of pathogens directly from clinical urines by MALDI-TOF

The optimised assay (see Figure 17 Section 2.2.2) was performed on 150 clinical urines. These comprised 129 mid-stream urines and 21 catheter-stream urines from 66 in- and 82 out- patients. The patient and sample characteristics were described in Table 16.

MALDI-TOF detected pathogens in 81 out of the 96 (84.3%) culture-positive samples (Table 17), identifying the same pathogen as culture in 69 cases (71.8%) (Table 18). Discrepancies were found between MALDI-TOF and conventional identification for the other 12 of these 81 (81 minus 69) culture-positive urines (Table 19).

MALDI-TOF did not identify bacteria in 15 out of the 96 culture-positive urines (Table 20); 8 out of these 15 failures related to low bacterial counts (< 10^5 cfu/mL), 3 to mixed bacterial growth (>2 species), 2 failures were with urines containing

particularly high numbers of WBCs or RBCs (> 10 000/µL) which might interfere with MALDI-TOF analysis, the last 2 failures remain unexplained.

MALDI-TOF also detected organisms in 8 out of the 27 culture-negative urines, in four cases detecting microorganisms (e.g. *Actinobacillus schaalii*, *Gardnerella vaginalis, Peptoniphilus harei*) unable to grow in routine media for urines (see Table 20) and in the other four bacteria found (*E. coli* and *E. faecalis*) that should have grown on routine media, but may have been recorded as insignificant growth (< 20 colonies/plate) or have been inhibited by antibiotics in the urine.

In 16 out of 27 urines that gave heavy mixed bacterial growth on culture, MALDI-TOF identified bacteria (Table 21) with 8 cases where two organisms were identified simultaneously.

The sensitivity of the technique was 84.4%; specificity was 70.0% but increased to 100% if the 8 organisms identified by MALDI-TOF from culture-negative urines were taken as true positives. Identification agreement was 70%. Overall, MALDI-TOF recognized 89 samples (59%) with mono-microbial infection and 16 (10%) with poly-microbial mixtures; (Table 17) these latter were categorized as mixed bacterial growth on culture, without species identification.

Table 16. Characteristics of the patients and their urine specimens used for MALDI-TOF identification assay.

Gender	Number of patients (n=150)	Age (years)
Female	91	1-94 (mean 61)
Male	59	6-97 (mean 74)

	Number of MSU	Number of CSU	Total
+ve culture	78	18	96
-ve culture	27	0	27
Mixed	24	3	27
Total	129	21	150

Number of human cell (x 10^6/L)	Number of urines with WBC	Number of urines with RBC	Number of urines with epithelial cells
0-100	53	124	141
100-500	44	14	19
500-1000	13	6	0
1000-2000	13	2	0
2000-5000	14	1	0
5000-10 000	7	1	0
10 000-55 000	6	2	0

Table 17. Agreement between culture and MALDI-TOF.

Taking culture +ve as reference 'gold standard'				Taking MALDI-TOF as true positive		
	Culture -ve	Culture +ve	Total	Culture -ve	Culture +ve	Total
MALDI-TOF +ve	8	81	89	0	81	81
MALDI-TOF -ve	19	15	34	27	15	42
Totals	27	96	123	27	96	123
Sensitivity	84.4%			84.4%		
Specificity	70%			100%		

	Mono-microbial infection found by MALDI-TOF	Poly-microbial mixtures found by MALDI-TOF	MALDI-TOF +ve/-ve results
+ve culture (n=96)	73	8	81/15
-ve culture (n=27)	8	0	8/19
Mixed culture (n=27)	8	8	16/11

Table 18. Identification agreement between culture and MALDI-TOF.

Pathogens	Culture total	MALDI-TOF total	I/D agreement
Escherichia coli	22	25	17
Pseudomonas aeruginosa	16	14	13
Enterococcus faecalis/faecium	15	14	12
Other Coliform species *Citrobacter freundii* *Citrobacter koseri* *Enterobacter cloacae* *Klebsiella pneumoniae* *Klebsiella oxytoca*	13*	10 1 1 3 4 1	9
Proteeae *Proteus mirabilis* *Providencia stuartii* *Morganella morganii*	11**	8 6 1 1	8
Streptococcus spp. (Group B)	8	5	5
Staphylococcus aureus	4	5	3
Staphylococcus saprophiticus	2	2	1
Candida albicans	1	1	1
Coag-Neg Staph	3	1	0
Streptococcus spp. (Group A)	1	2	0
Acinobacillus schaalii	0	4	0
Clostridium spp.	0	1	0
Gardnerella vaginalis	0	2	0
Lactobacillus spp.	0	2	0
Peptoniphilus harei	0	1	0
SUM	**96**	**97**	**69**

* As based on Chrom ID culture: split as *Citrobacter* spp., *Enterobacter* spp., *Klebsiella* spp. only by MALDI-TOF.

** As based on Chrom ID culture: split as *Morganella morganii, Proteus mirabilis, Providencia stuartii* by MALDI-TOF.

Table 19. Disagreements between positive culture and MALDI-TOF bacterial identification.

Urine	Found by Culture	Found by MALDI-TOF
	Gram-negative bacteria able to grow on standard media	
1	*E. coli*	*E. aerogenes*
2	Other Coliform species *	*E. coli*
3	Other Coliform species *	*E. coli*
4	Proteeae**	*P. aeruginosa*
5	*S. saprophyticus*	*E. coli*
	Gram-positive bacteria able to grow on standard media	
6	Coag-Neg Staph	*S. aureus*
7	*Enterococcus* spp.	*S. epidermidis*
8	Coag-Neg Staph	*S. saprophyticus*
9	Other coliform species	*Clostridium* spp.
	Bacteria difficult to cultivate	
10	Coag-Neg Staph	*Acinobacullum schallii*
11	*P. aeruginosa*	*Acinobacullum schalii*
12	*P. aeruginosa*	*Acinobacullum schalii*

**Citrobacter* spp., *Enterobacter* spp., *Klebsiella* spp.

***Morganella morganii, Providencia stuartii, Proteus mirabilis*

Table 20. Summary of MALDI-TOF disagreements.

MALDI -TOF -ve *versus* +ve culture (n=15)	
Bacterial count below detection limit (<10^5 /mL)	8
Heavy-mixed bacterial growth on culture	3
High number of RBCs or WBCs (>10 000/µl)	2
Unknown reason	2
MALDI-TOF +ve *versus* -ve culture (n=8)	
Aerobic bacteria able to grow on standard medium	
E. coli (n=3) *E. faecalis* (n=1)	
Anaerobic bacteria difficult to cultivate	
Gardnerella vaginalis (n=2) *Acinobaculum schalii* (n=1) *Peptoniphilus harei* (n=1)	

Table 21. Organisms detected in mixed cultures by MALDI-TOF.

Single organisms found(n=8)	Two organisms found (n=8)
E. coli (n=3)	*E. coli* + *Citrobacter amalonaticus*
K. oxytoca	*E. coli* + *Citrobacter farmeri*
P. mirabilis	*Enterobacter kobei* + *Enterobacter asburiae*
E. faecalis	*K. pneumoniae* + *E. coli*
Lactobacillus jensenii (n=2)	*K. oxytoca* + *Roultella ornithicolytica*
	Streptococcus dysgalactiae + *S. marcescens*
	Streptococcus castoreus + *Lactobacillus jensenii*
	E. faecalis + *Enterobacter* spp.

Mixed cultures means more than 2 bacteria growth on the plate by clinical laboratory, which did not identify the organisms in these circumstances.

3.2.3 Detection of β-lactamase activity directly from urine by MALDI-TOF

The main objective of this section was to detect hydrolysis of cephalosporins directly from urines infected by cephalosporin-resistant bacteria, without culture.

3.2.3.1 Optimising detection of cephalosporin-hydrolysing activity

We sought to optimise sample preparation to detect hydrolysis of cephalosporins by ESBLs and AmpC producers directly from clinical urines. For this purpose it was first necessary to show that cephalosporins were stable in a buffer solution suitable for MALDI-TOF analysis and then to assess the best conditions to detect hydrolysis.

Antibiotic solution buffer

Among three tested buffer solutions (10 mM ammonium phosphate pH 7.2, 10 mM ammonium bicarbonate pH 8-9 and 10 mM ammonium hydrogen citrate pH 7.2) only ammonium hydrogen citrate allowed both stable and long-term storage of cefotaxime and ceftazidime stock concentration and examination of the hydrolysis of these antibiotics by MALDI-TOF.

Incubation time

MALDI-TOF generally could detect hydrolysis if cultivated isolates were suspended in cephalosporin solution in ammonium hydrogen citrate and incubated for 30 minutes (cefotaxime 0.5 mg/mL and ceftazidime 0.25 mg/mL), one hour (cefepime 0.5 mg/mL) and 1.5 hours (cefpodoxime 0.25 mg/mL, ceftriaxone 0.5 mg/mL) when using *E. coli* strain H141480453 harbouring CTX-M-15, NDM-4 and OXA-181 β-lactamases or *E. coli* strain J53 producing TEM-10 β-lactamase (only ceftazidime) at 37^{o}C with shaking at 900 rpm.

The exception was that the *E. coli* H141480453 positive control found to give an implausible results for ceftazidime perhabs because its enzymes are kinetically inefficient for this cephalosporin (Nordmann *et al.*, 2009).

Although hydrolysis was detectable within 30 min we allowed for strains with low levels of enzyme, by adopting a standard 2-hour incubation for all antibiotics except ceftazidime where this was extended to 4 hours. The results for all tested cephalosporins with incubation for different time period is presented in Figures 50, 51, 52, 53 and 54.

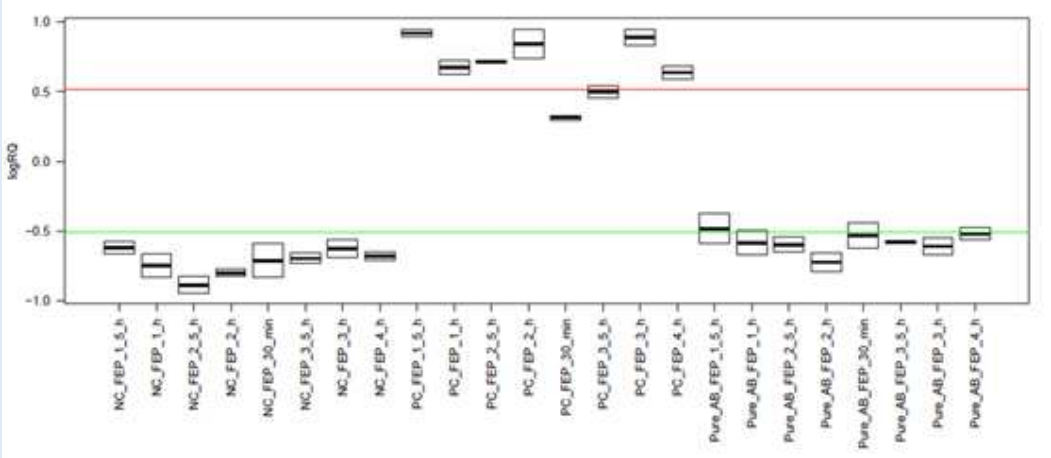


Figure 50. Optimization of incubation time for cefepime using the prototype MBT STAR-BL hydrolysis assay.

Legend: NC_FEP_1.5_h- β-lactamase -ve control incubated for 1.5 h with cefepime
PC_FEP_1.5_h- CTX-M-15 +ve control incubated for 1.5 h with cefepime
Pure AB_FEP_1.5_h- cefepime in buffer, incubated for 1.5 h
e.g. here '30 , 1, 1.5, 2, 2.5, 3, 3,5, 4_h' means incubated for 30 min, 1h, 1.5h, 2h, 2.5h, 3h, 3.5h, 4h.
logRQ (logarithm RQ)- resistance quotient.

Boxes show the range of peak intensities collected from 800 laser shots from different positions of the target spot with the thick mid bar the mean.

Review of the peak patterns for the non-hydrolysed and the hydrolysed forms of the different cephalosporins led to different of characteristic profiles for β-lactamase producing and non-producing strains. Changes in the peak intensity ratios within a spectrum allowed quantitative evaluation of hydrolysis, measured as logarithm RQ (resistance quotient).

Isolates were classified as negative (below green threshold) if the peak intensity distributions corresponding to the non-hydrolysed cephalosporins and were similar to those for the negative control. Isolates were classified as positive (above red threshold) if the intensity of peaks corresponding to the hydrolysed cephalosporin were similar to those for the positive control. Isolates showing an intensity distribution between the negative and positive controls were called 'slow hydrolysers'.

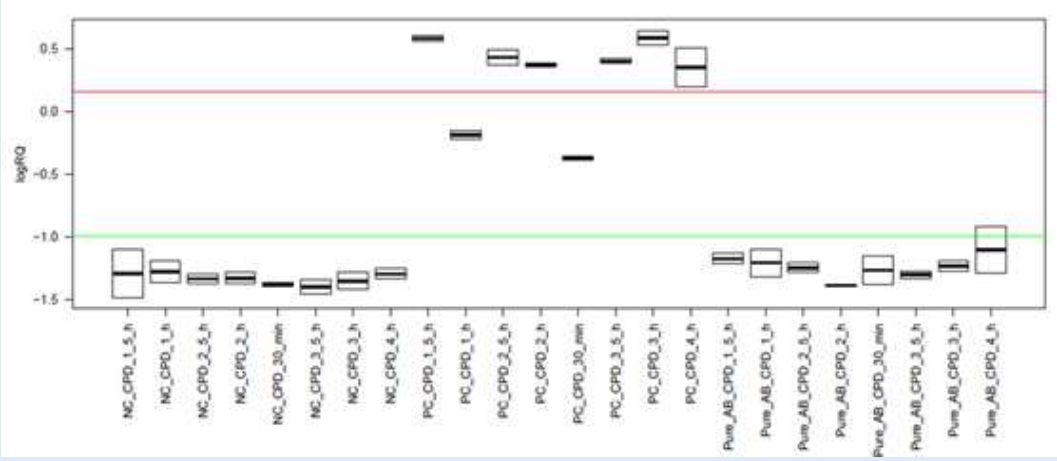


Figure 51. Optimization of incubation time for cefpodoxime using the prototype MBT STAR-BL hydrolysis assay.

Legend: NC_CPD_1.5_h- β-lactamase -ve control incubated for 1.5 h with cefpodoxime
PC_CPD_1.5_h- CTX-M-15 +ve incubated for 1.5 h with cefpodoxime
Pure AB_CPD_1.5_h- cefpodoxime in buffer, incubated for 1.5 h
e.g. here '30, 1, 1.5, 2, 2.5, 3, 3,5, 4_h' means incubated for 30 min, 1h, 1.5h, 2h, 2.5h, 3h, 3.5h, 4h'.
logRQ (logarithm RQ)- resistance quotient.

Interpretation of results was as for Figure 50.

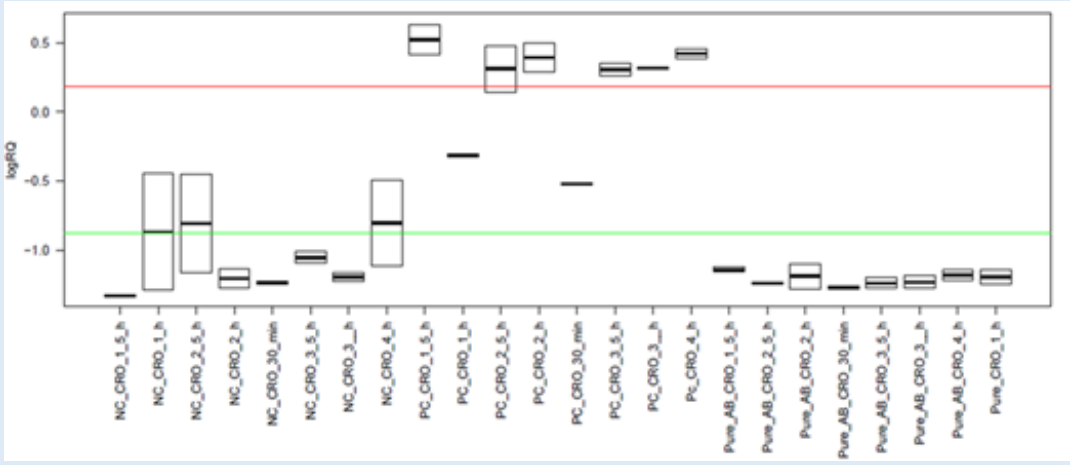


Figure 52. Optimization of incubation time for ceftriaxone using the prototype MBT STAR-BL hydrolysis assay.

Legend: NC_CRO_1.5_h- β-lactamase -ve control incubated for 1.5 h with ceftriaxone
PC_CRO_1.5_h- CTX-M-15 +ve control incubated for 1.5 h with ceftriaxone
Pure AB_CRO_1.5_h- ceftriaxone in buffer, incubated for 1.5 h
e.g. here '30, 1, 1.5, 2, 2.5, 3, 3,5, 4_h' means incubated for 30 min, 1h, 1.5h, 2h, 2.5h, 3h, 3.5h, 4h'; logRQ (logarithm RQ)- resistance quotient.
Interpretation of results was as for Figure 50.

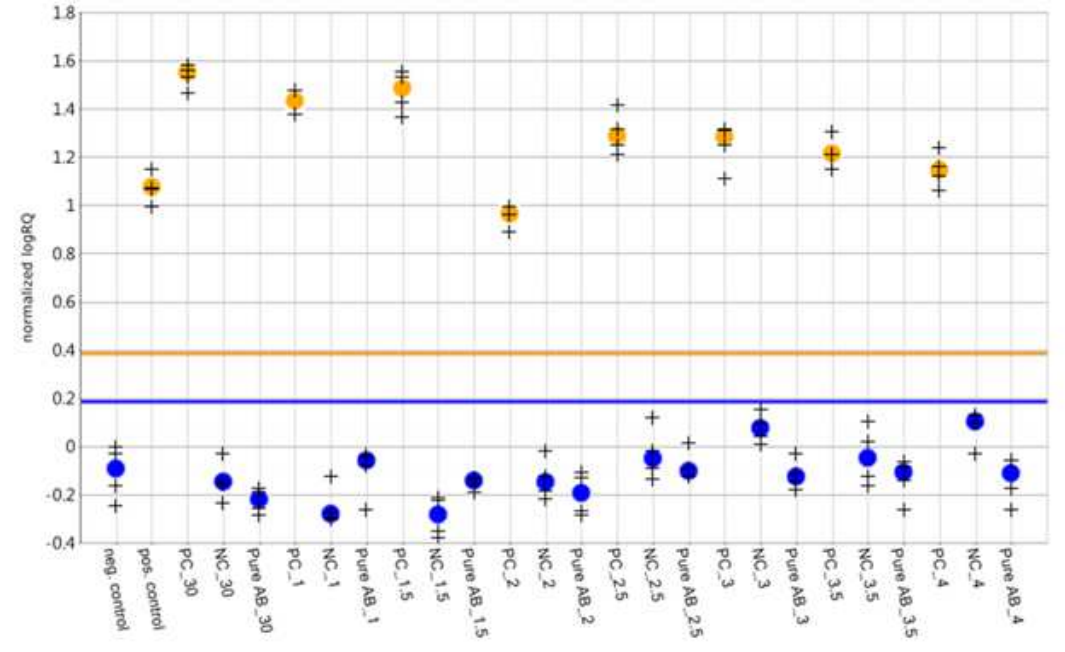


Figure 53. Optimization of incubation time for ceftazidime using Brüker's commercial MBT STAR-BL hydrolysis software.

Legend: neg. control- β-lactamase -ve control for ceftazidime.
pos. control- TEM-10 +ve control for ceftazidime.

PC_30- TEM-10 +ve control for ceftazidime, incubated for 30 min
NC_30- β-lactamase -ve control for ceftazidime, incubated for 30 min
Pure AB_30- ceftazidime in buffer, incubated for 30 min
e.g. here '30, 1, 1.5, 2, 2.5, 3, 3,5, 4_h' means incubated for 30 min, 1h, 1.5h, 2h, 2.5h, 3h, 3.5h, 4h'.
normalized logRQ (logarithm RQ)- resistance quotient.

Isolates were classified as negative (blue and below the blue line) if the peak intensity distributions corresponding to the non-hydrolysed cephalosporins and were similar to those for the negative control.

Isolates were classified as positive (orange and above the orange line) if the intensity of peaks corresponding to the hydrolysed cephalosporin were similar to those for the positive control.

Isolates showing an intensity distribution between the negative and positive controls were called 'slow hydrolysers'.

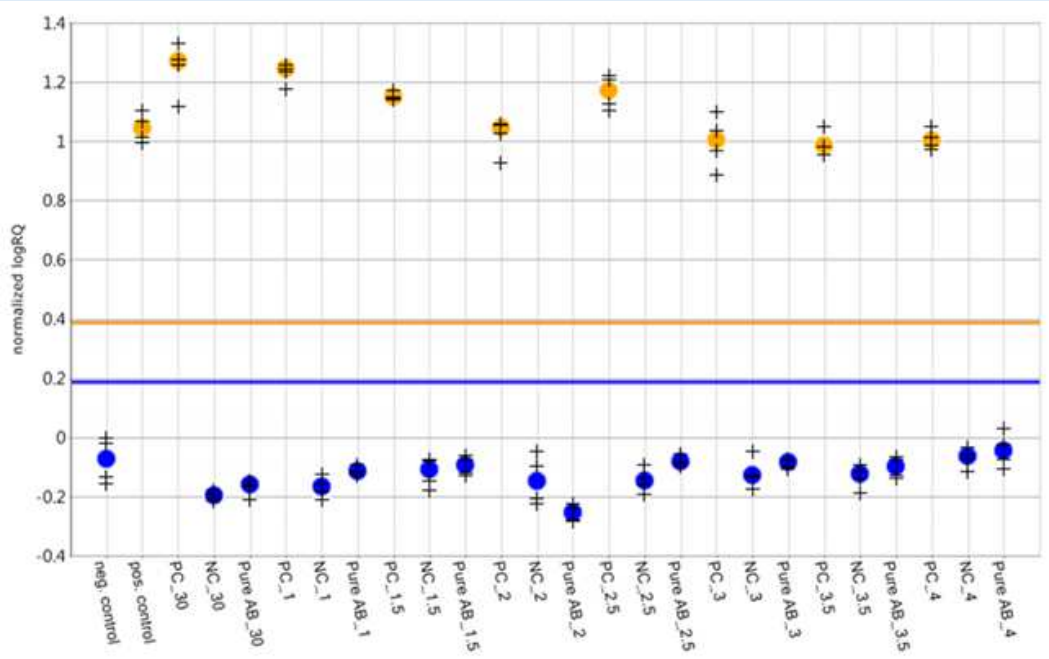


Figure 54. Optimization of incubation time for cefotaxime using commercial MBT STAR-BL Bruker's hydrolysis software.

Legend: neg. control- β-lactamase -ve control for cefotaxime, incubated for 30 min
pos. control- CTX-M-15 +ve control for cefotaxime, incubated for 30 min
PC_30- CTX-M-15 +ve control for cefotaxime, incubated for 30 min
NC_30- β-lactamase -ve control for cefotaxime, incubated for 30 min
Pure AB_30- cefotaxime in buffer, incubated for 30 min
e.g. here '30, 1, 1.5, 2, 2.5, 3, 3,5, 4_h' means incubated for 30 min, 1h, 1.5h,
2h, 2.5h, 3h, 3.5h, 4h'.
normalized logRQ (logarithm RQ)- resistance quotient calculation.

Interpretation of results was as for Figure 53.

3.2.3.2 Spectra of native and hydrolysed cephalosporins

MALDI-TOF was used to detect cephalosporinase activity in infected urines. β-Lactamases hydrolyse the β-lactam ring of penicillin and cephalosporin antibiotics. Mass spectrometry detects molecules based on their molecular mass and it would be predicted that hydrolysis would correspond to disappearance of native peak patterns and the appearance of peaks corresponding to the hydrolysed forms of the cephalosporins. This might be exploited to distinguish rapidly β-lactamase-producing and -non-producing strains.

Cefepime

Analysis of cefepime in buffer or after incubation for 2 hours with a β-lactamase-negative control susceptible to cefepime, showed the presence of a peak $[M + H]^+$ at 481.43 Da. This value corresponds to the native cefepime molecule (480.56 g/mol). When cefepime was incubated with the CTX-M-15 positive control for 2 hours this peak disappeared. A second peak $[M\text{-}Z^1 + H]^+$ at 396 Da was also detected with native cefepime and retained during incubation with the β-lactamase -ve control but likewise disappeared during incubation with CTX-M-15 producer that was presented in Figure 55.

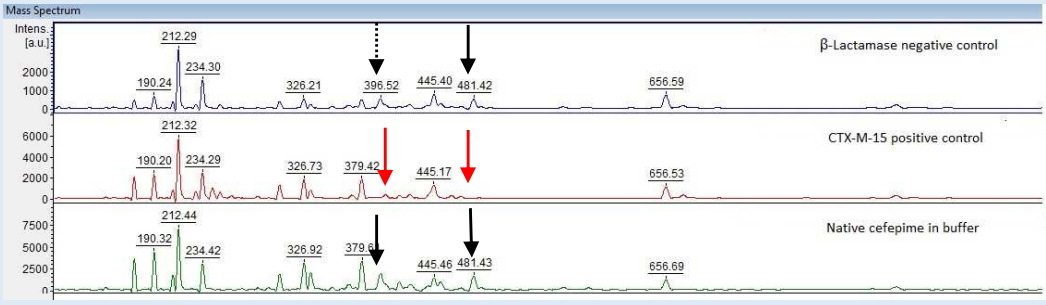


Figure 55. Mass spectra for native cefepime and cefepime incubated for 2 hours with β-lactamase- positive and -negative controls.

↓ - indicates peaks lost during incubation with CTX-M-15

↓ - indicates peaks associated with native cefepime

[1] 1-methyl-pyrrolidin group, belonging to the cefepime structure.

Ceftriaxone

A peak of the expected molecular mass (554.58 g/mol) was seen for the native ceftriaxone in buffer and when it was incubated for 2 hours with the β-lactamase-negative control at $[M + H]^+$ 555.29 Da and 556.22 Da, respectively, but it was lost after 2 hours incubation with the β-lactamase-positive (CTX-M-15) control. Other peaks, at $[M\text{-}X^2+H]^+$ 396 Da, $[M+Na]^+$ 577 Da and 621 Da also were lost during incubation with CTX-M-15 but retained with the native ceftriaxone in buffer and when the antibiotic was incubated with ceftriaxone -susceptible bacteria (see Figure 56).

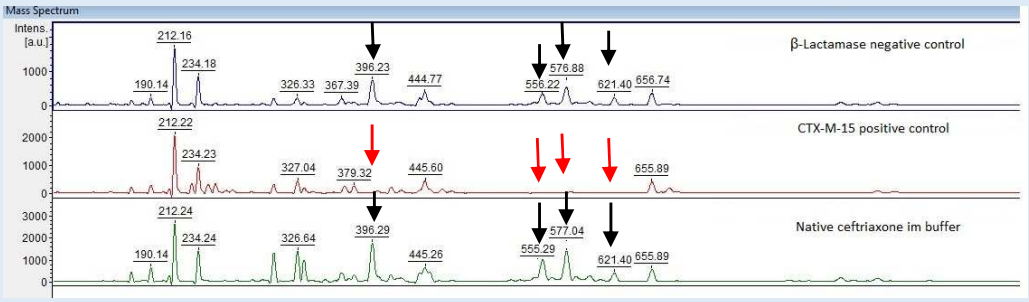


Figure 56. Mass spectra for native ceftriaxone and ceftriaxone incubated for 2 hours with β-lactamase-positive and -negative controls.

↓- indicates peaks lost during incubation with CTX-M-15

↓- indicates peaks associated with native ceftriaxone

Cefpodoxime

Native cefpodoxime (427.46 g/mol) gave peaks at $[M + H]^+$ 428.49 Da. These peak remained when cefpodoxime was incubated with the β-lactamase-negative control but they were lost with the CTX-M-15 β-lactamase-positive control (see Figure 57).

[2] Triazine-ylthiol group, belonging to the cefotaxime structure.

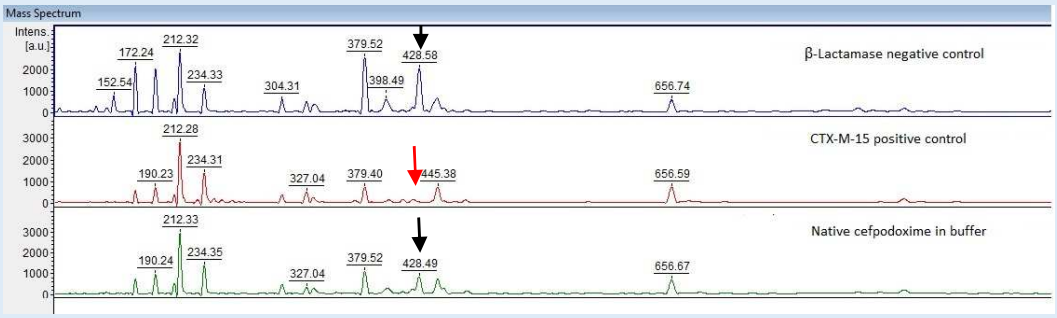


Figure 57. Mass spectra for native cefpodoxime and cefpodoxime incubated for 2 hours with β-lactamase-positive and -negative controls.

↓- indicates peak lost during incubation with CTX-M-15

↓- indicates peak associated with native cefpodoxime

Cefotaxime

In the case of cefotaxime (455.47 g/mol) peaks at $[M + H]^+$ 456.35 Da and $[M + Na]^+$ 478 Da were seen and retained during incubation with the β-lactamase-negative control. These peaks were lost during incubation with the β-lactamase-positive (CTX-M-15) control, as presented in Figure 58.

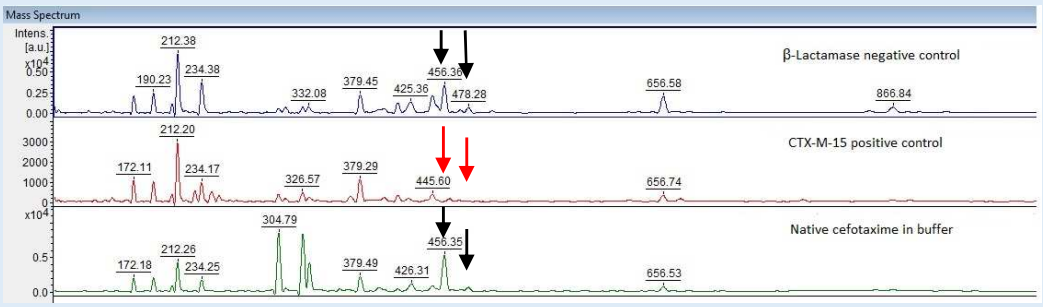


Figure 58. Mass spectra for native cefotaxime and cefotaxime incubated for 2 hours with β-lactamase-positive and -negative controls.

↓ - indicates peaks lost during incubation with CTX-M-15

↓ - indicates peaks associated with native cefotaxime

Ceftazidime

Mass spectrometry of native caftazidime (546.58 g/mol) detected two peaks at $[M + H]^+$ 547 Da and $[M\text{-}Y^3 + H]^+$ 468.89 Da that were retained during incubation with the β-lactamase-negative control, but were lost during incubation with a TEM-10 β-lactamase-positive control. Incubation with the latter enzyme was also associated with the appearance of two peaks at 461.63 Da and 152 Da as shown in Figure 59.

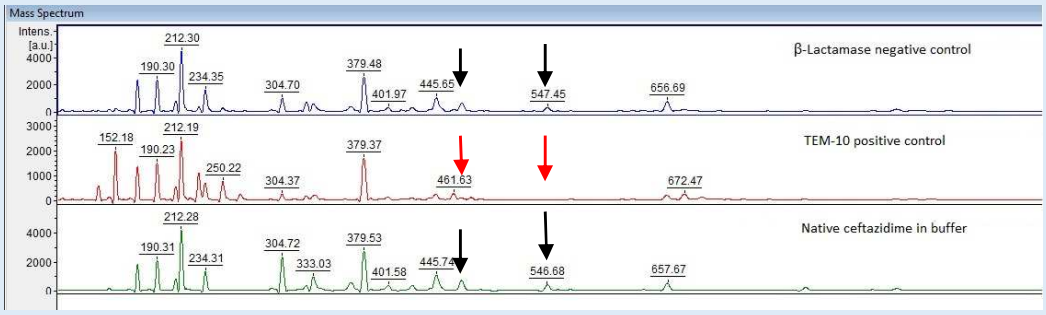


Figure 59. Mass spectra for native ceftazidime and ceftazidime incubated for 4 hours with β-lactamase-positive and -negative controls.

↓- indicates peaks lost during incubation with TEM-10

↓- indicates peaks associated with native ceftazidime

Non-cephalosporin-related peaks

Peaks associated with the HCCA (α-cyano-4-hydroxy-cinnamic acid) matrix ($[M + H]^+$ 189.17 Da) and ($[2M + H]^+$ 379.02 Da) were seen when cephalosporin was incubated with hydrolytic and non-hydrolytic isolates and with the cephalosporins in buffer with peaks at 190 Da and 379 Da (Figure 55, 56, 57, 58, 59). Other peaks at: 172, 207, 212, 234, 304, 326, 332, 445, 656 Da (Table 22) were seen also with pure cephalosporins in buffer and with the susceptible and resistant controls added, probably indicating the presence of other components in the matrix. Two 'small' peaks at 227 Da and 250 Da were present only when bacteria, whether

[3] Pyridine group, belonging to the ceftazidime structure.

cephalosporin-hydrolysing or not were added; these probably arose from bacterial components.

Table 22. Peaks visable after incubation of cephalosporins with β-lactamase-positive, and -negative controls and for native cephalosporins in buffer.

Mass range	FEP			CRO			CPD			CTX			CAZ		
	PC[1]	NC	PA	PC[1]	NC	PA	PC[1]	NC	PA	PC[1]	NC	PA	PC[2]	NC	PA
152-153							-	+	-				+	+	-
172-172.50	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
190-190.50	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
207-207.50	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
212-212-50	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
227-228.50	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-
234-234.50	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
250-250.50	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-
304-305	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
326-327	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
332-333	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
367-368.50				+	+	+									
379-380.50	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
396-398	-	**+**	**+**	-	**+**	**+**	+	+	+						
413-414										+	+	+			
425-426										+	+	+			
428-429							-	**+**	**+**						
445-445.50	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
456-457										-	**+**	**+**			
461-462													+	+	-
468-469													-	**+**	**+**
478-479										-	**+**	**+**			
481-481.50	-	**+**	**+**												
547-548													-	**+**	**+**
554-555				-	**+**	**+**									
577-578				-	**+**	**+**									
621-622				-	**+**	**+**									
656-657	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Legend: PC[1]- β-lactamase-positive control (CTX-M-15), PC[2]- β-lactamase-positive control (TEM-10), NC- β-lactamase-negative control, PA- pure antibiotic;

+ peak detected, - peak invisible, ■ - peak seen for native cephalosporin in buffer and when incubated with the β-lactamase-negative control but lost when the cephalosporin as incubated with the β-lactamase-positive control.

3.2.3.3 Detection of cephalosporin-hydrolysing activity directly from urine and cultivated bacteria

The purpose of this study was to detect cephalosporin hydrolysis, as reflected in the loss of the peaks highlighted orange in Tables 22 when cultivated bacteria or those harvested from clinical urines were incubated with native cephalosporins.

Decreased susceptibility to cephalosporins in Enterobacteriaceae is mostly due to ESBL production but can also involve plasmid-mediated AmpC, overproduction of the chromosomal AmpC β-lactamase or metallo and KPC enzymes. All these enzymes cause hydrolysis and phenotypic resistance to 3rd and (except AmpC), 4rd generation cephalosporins.

Assays were performed on 91 urines and the 91 bacterial isolates cultured from these urines (see Table 23). Forty-three out of the 91 were ESBL producers based on phenotypic testing performed by the NNUH clinical laboratory, including *E. coli* (n=40) and *K. pneumoniae* (n=3); 22 were high-level AmpC β-lactamase producers compromising *C. freundii* (n=1), *C. braakii* (n=1), *E. cloacae* (n=2), *E. aerogenes* (n=4), *E. coli* (n=12), *K. pneumoniae* (n=1) and *S. marcescens* (n=1); finally 26 isolates were *E. coli* that were fully susceptible to cephalosporins (Table 23).

Cephalosporin-hydrolysis results in term of peak profile were reviewed for all tested urines and their bacterial isolates in two ways (i) in relation to phenotypic susceptibility data for each of the cephalosporins (Table 24, 25, 26) and (ii) in relation to β-lactamase genes found by sequencing (Table 33). Calculations for sensitivity and specificity for each of the cephalosporin assays are presented in detail in the Appendix B. Detection of cephalosporin hydrolysis was based on the analysis by automated and manual softwares (see Sections 2.2.3.2 and 2.2.3.3) *vs.* phenotypic results. Results with all urines tested directly are presented in Table 27 and those from the cultivated isolates in Table 28. Table 29, separately details ESBL producers and Table 30 AmpC producers.

Table 23. Number of urines collected from clinical laboratory for cephalosporin-hydrolysis assay by MALDI-TOF and sequencing.

Yielding ESBL-producers by phenotypic testing according to the D68C test	43
No. of these ESBL isolates sequenced	41
No. confirmed to have ESBLs by sequencing	38
No. not sequenced	2
No. with no ESBL not found by sequencing	3
Yielding AmpC producers by phenotypic testing according to the D68C test	**22**
No. of these AmpC isolates sequenced	10
No. with plasmid AmpC found by sequencing	4
Sequenced *Enterobacter* spp./*Citrobacter* spp. inferred from phenotype to have derepressed chromosomal AmpC	6
No. not sequenced	12
No of fully susceptible urines collected from clinical laboratory	**26**
Total	**91**

Cefepime assay

Cefepime hydrolysis was detected for 39 out of the 50 urines containing cefepime-resistant bacteria (Table 27); 38 of these had ESBLs and one had an AmpC enzyme and gave an intermediate zone in a disc test (Tables 29, 30). No hydrolysis was found for 39 urines; 35 containing bacteria with phenotypic susceptibility to cefepime and 4 resistant (3 of these latter were AmpC producers with zones of 11-24 mm *vs.* a resistant breakpoint ≤ 26 mm based on BSAC criteria) (Tables 27, 29, 30). Slow hydrolysis was detected for 11 urines; 2 of these containing bacteria with ESBLs and 8 had an AmpC enzyme (Tables 27, 30). The assay gave unreliable results for 2 urines containing bacteria with ESBL enzyme based on disagreements between the peak profiles acquired by the two softwares (Tables 27, 29). The sensitivity and specificity of the assay in relation to cefepime was 91% and 87% taking intermediate zones as resistant (Table 27).

Assays with the cultivated isolates gave higher sensitivity (98%), but lower specificity (69-71%) compared with urines (Table 28). Hydrolysis was detected in 49 (42 ESBLs producers, 7 AmpC producers) out of the 50 cefepime-resistant isolates

(Tables 28, 29, 30). Five further isolates produced AmpC enzymes gave also positive results though they had susceptible (n=4) and intermediate phenotypic profiles (n=1) (Tables 28, 30). No hydrolysis was seen for 28 isolates and 27 of these had susceptible phenotypic profiles. Slow-hydrolysis was found for seven cefepime-susceptible isolates; 6 of these had an AmpC enzyme. No reliable results were obtained for two cefepime susceptible isolates, which were classified as cefepime-hydrolysing by automated software but with a visible peak $[M + H]^+$ retained at 480.50 Da as examined by manual software (Table 28).

Ceftriaxone assay

Hydrolysis was found for 40 out of the 54 urines containing ceftriaxone-resistant bacteria including 39/43 ESBL producers (Tables 27, 29). No hydrolysis was detected for 47 urines: these compromised 30 out of the 31 urines containing bacteria with ceftriaxone-susceptible profiles, 5 intermediate isolates and 12 that were resistant (10 of these were AmpC producers) (Tables 27, 30). Slow-hydrolysis was detected for one urine containing bacteria with ESBL profile. No reliable result was found for one ESBL- positive urine, where manual data analysis suggested loss of the ceftriaxone peak and agreed with phenotypic resistance, but the assay was classified as negative by the automated software analyses (Tables 27, 29). The assay failed for two AmpC-positive urines where no peaks were identified (Tables 27, 30). Overall, the ceftriaxone assay showed high specificity (100%), but low sensitivity (70.7-77.4%) (Table 27).

Ceftriaxone hydrolysis assays with cultivated isolates showed slightly higher sensitivity at 83.3-88.9%, than with urine assay and 100% specificity, taking six intermediate isolates as resistant. Hydrolysis was detected for 45 out of the 54 ceftriaxone-resistant isolates and one intermediate (included 42/43 ESBLs producers) (Tables 28, 29). No hydrolysis was found with 41 isolates of which 31 were ceftriaxone susceptible, four were intermediate and six were ceftriaxone-resistant with these including five producing AmpC β-lactamases. Slow-hydrolysis was detected for five isolates with AmpC enzyme (Tables 28, 30).

Cefpodoxime assay

Using urines directly, hydrolysis was detected with 45 out of the 65 urines containing bacteria resistant to cefpodoxime; 39 of these producers had ESBL and 6 AmpC (Tables 27, 29, 30). No hydrolysis was found for 35 urines, including 25 that had cefpodoxime-susceptible bacteria and 10 cefpodoxime-resistant; 8 of these latter 10 had AmpC enzymes (Tables 27, 30). Slow-hydrolysis was seen for 9 urines containing bacteria with cefpodoxime resistance (7 AmpC and 2 ESBLs). The assay failed for 2 urines (Tables 27, 29, 30).

Using isolates, hydrolysis was detected with 56 out of the 65 cefpodoxime-resistant isolates; 42 of these had ESBLs and 14 had AmpC (Tables 28, 29, 30). No hydrolysis was found for 24 isolates, in agreement with their phenotypic profiles and for 3 resistant isolates, all of them producing AmpC enzyme. Slow hydrolysis was seen for 5 isolates producing AmpC, one isolate producing ESBL and one isolate with susceptible profile (Tables 28, 30). Sensitivity using urines and isolates was 84.4% and 95.4%, respectively. Specificity was 100% for urines and slightly lower at 96 % for cultivated isolates (Table 28).

Ceftazidime assay

Hydrolysis was detected with 27 out of the 61 urines containing bacteria resistant to ceftazidime. These 27 urines contained ESBL producers (Tables 27, 29). No hydrolysis was found with 21 urines out of the 30 containing bacteria susceptible to ceftazidime but also for 30 out of the 61 urines containing bacteria resistant to ceftazidime (13 ESBL producers and 17 with AmpC). Slow-hydrolysis was detected for 3 urines containing bacteria with phenotypic resistance and one with susceptible profile. The assay failed for nine urines. Sensitivity of the assay was low (49.2%) although specificity was higher (91.3%) (Table 27).

Using cultivated bacteria, ceftazidime hydrolysis was detected with 47 out of the 61 ceftazidime-resistant isolates; 39 of these were ESBL producers and 8 were AmpC

producers (Tables 28, 29, 30). No hydrolysis was detected for 40 isolates, including 29 that had a ceftazidime-susceptible phenotype and 11 were resistant; 8 out of these latter 11 produced AmpC β-lactamase enzymes, and gave zones 14-22 mm (Tables 28, 30). Slow hydrolysis was found for four isolate; 3 of these with phenotypic resistance to ceftazidime. Sensitivity and specificity of the isolate assay was higher than for urines (82%, 96.7%, respectively).

Cefotaxime assay

Hydrolysis was found with 39 out of the 60 urines containing bacteria resistant to cefotaxime including 39 with ESBL producers (Tables 27, 29). No hydrolysis was detected for 25 out of the 29 urines containing bacteria susceptible to cefotaxime and 12 containing bacteria resistant to cefotaxime, including all AmpC producers (Tables 27, 30). Slow hydrolysis was seen for 13 urines containing bacteria producing ESBLs (n=3) and AmpC (n=8). No reliable results were found for 2 urines. The sensitivity of the assay was in a range 79.7.3%-80.3% and specificity was in a range 83.3%-89.3% (Table 27).

Hydrolysis was detected with 47 out of the 60 cultured isolates resistant to cefotaxime; 40 of these produced ESBLs and 7 had AmpC (Tables 28, 29, 30). No hydrolysis was seen for 28 isolates that were ceftazidime susceptible with agreement to their susceptibility test results, also for 6 AmpC producers and one ESBL producer resistant to cefotaxime (Tables 28, 30). No reliable results were obtained for 4 isolates. Slow hydrolysis was seen for five AmpC producers. Sensitivity of the assay with isolates was higher than for urines (87.5%-87.9%) and specificity was in a range 90.3-96.7% (Table 28).

Overall, both urines containing ESBL producers and cultivated ESBL isolates from these urines typically achieved full hydrolysis for all 5 cephalosporins tested (25/43 for urines; 36/43 for isolates with most of the remainder hydrolysing 4 cephalosporins) whereas hydrolysis for AmpC producers was unreliably detected for both urines and isolates (see Tables 24, 25, 30). Bacteria with cephalosporin-susceptible phenotypic profiles did not show full hydrolysis for any cephalosporins, however slow hydrolysis was seen for seven cephalosporins using both urines (n=4) and isolates (n=3).

Cephalosporin hydrolysis assays showed similar results for ESBL producers using both bacterial isolates and urines (Table 29). Using urines hydrolysis was detected in over 90% tests for all cephalosporins (39/43 ESBL producers) except ceftazidime, where hydrolysis was seen with 62.8% (27/43 ESBL producers). Using the corresponding isolates hydrolysis was detected in 91%-98% of tests for all cephalosporins (see Tables 25, 29). Hydrolysis was less reliably detected with AmpC producers, reflecting slow hydrolysis. Assays using urines containing bacteria producing AmpC enzyme did not reliably detect hydrolysis for any cephalosporin except cefpodoxime in where it was detected for 6 out of 22 cefpodoxime-resistant cases. Using isolates hydrolysis was detected for 14/22 AmpC producers (Tables 25, 30).

The overall cephalosporins sensitivity of the assays for ESBL producers from urines and their cultivated isolates was 91.4% and 98.1%, respectively whereas for AmpC producers sensitivity was significantly lower for both urines and bacterial isolates (32.4% and 69.7%, respectively) (see Tables 29, 30 and B6, B7 Appendix B).

Table 24. Numbers of cephalosporins hydrolysed by urines and corresponding isolates in relation to isolate phenotypes and genotypes.

Number of urines or isolates achieving full hydrolysis of indicated number of cephalosporins						
No. cephalosporins	**5**	**4**	**3**	**2**	**1**	**0**
Urines tested directly						
Containing sequenced ESBL producers (n=38)	22	11	1	1	0	3
Containing unsequenced ESBL producers (n=2)	1	1	0	0	0	0
Containing ESBL producers not confirmed by sequencing (n=3)	2	1	0	0	0	0
Containing sequenced isolates with plasmid AmpC (n=4)	0	0	0	1	2	1
Containing sequenced AmpC derepressed Enterobacter/Citrobacter (n=6)	0	0	0	0	2	4
Containing unsequenced AmpC isolates (n=12)	0	0	0	0	2	10
Urines containing cephalosporin-susceptible bacteria (n=26)	0	0	0	0	0	26
Isolates cultured from urines						
Sequenced ESBL producers (n=38)	32	4	2	0	0	0
Unsequenced ESBL producers (n=2)	1	1	0	0	0	0
ESBL producers not confirmed by sequencing (n=3)	3	0	0	0	0	0
Sequenced isolates with plasmid AmpC (n=4)	0	0	2	0	2	0
Sequenced AmpC derepressed Enterobacter/Citrobacter (n=6)	1	1	1	1	1	1
Unsequenced AmpC isolates (n=12)	0	1	4	1	3	3
Cephalosporin-susceptible bacteria (n=26)	0	0	0	0	0	26

Table 25. Total number of urines and isolates cultured from these urines achieving full hydrolysis for particular cephalosporins.

	Number of urines or isolates achieving full hydrolysis				
	FEP	**CRO**	**CPD**	**CAZ**	**CTX**
Urines containing sequenced ESBL producers (n=38)	33	34	34	24	34
Sequenced isolates producining ESBL (n=38)	37	37	38	35	35
Urines containing unsequenced ESBL producers (n=2)	2	2	2	1	2
Unsequenced isolates producining ESBL (n=2)	2	2	1	2	2
Urines containing ESBL producers not confirmed by sequencing (n=3)	3	3	3	2	3
Isolates producing ESBL not confirmed by sequencing (n=3)	3	3	3	2	3
Urines containing sequenced plasmid AmpC producers (n=4)	0	1	3	0	0
Sequenced isolates with plasmid AmpC (n=4)	4	0	2	2	0
Urines containing sequenced AmpC derepressed Enterobacter/Citrobacter (n=6)	0	0	2	0	0
Sequenced isolates with AmpC derepressed Enterobacter/Citrobacter (n=6)	4	1	4	3	3
Urines containing unsequenced AmpC (n=12)	1	0	1	0	0
Unsequenced isolates producing AmpC (n=12)	4	2	8	3	4
Urines containing unsequenced cephalosporin-susceptible bacteria (n=26)	0	0	0	0	0
Unsequenced isolates susceptible to cephalosporin (n=26)	0	0	0	0	0

Table 26. Cephalosporin hydrolysis assays by MALDI-TOF versus phenotypic susceptibility testing.

No	Species & Mechanism	Methods	FEP	CRO	CPD	CAZ	CTX
1	*E. coli* FS	Disc diffusion	S	S	S	S	S
		Hydrolysis- urine	NH	NH	NH	NR	NH
		Hydrolysis- isolate	NH	NH	NH	NH	NH
2	*E. coli* FS	Disc diffusion	S	S	S	S	S
		Hydrolysis- urine	NH	NH	NH	NR	NH
		Hydrolysis- isolate	NH	NH	NH	NH	NH
3	*E. coli* FS	Disc diffusion	S	S	S	S	S
		Hydrolysis- urine	NH	NH	NH	NR	NH
		Hydrolysis- isolate	NH	NH	NH	NH	NH
4	*E. coli* FS	Disc diffusion	S	S	S	S	S
		Hydrolysis- urine	NH	NH	NH	NR	NH
		Hydrolysis- isolate	NH	NH	NH	NH	NH
5	*E. coli* FS	Disc diffusion	S	S	S	S	S
		Hydrolysis- urine	NH	NH	NH	NR	NH
		Hydrolysis- isolate	NH	NH	NH	NH	NH
6	*E. coli* ESBL	Disc diffusion	R (14)	R	R	R	R
		Hydrolysis- urine	H	H	H	NR	H
		Hydrolysis- isolate	H	H	H	H	H
7	*E. coli* ESBL	Disc diffusion	R (23)	R (15)	R	R (19)	R (14)
		Hydrolysis- urine	H	H	H	NH	H
		Hydrolysis- isolate	H	H	H	NH	H
8	*E. coli* FS	Disc diffusion	S	S	S	S	S
		Hydrolysis- urine	NH	NH	NR	NH	NH
		Hydrolysis- isolate	NH	NH	NH	NH	NH
9	*E. coli* FS	Disc diffusion	S	S	S	S	S
		Hydrolysis- urine	NH	NH	NH	NR	NH
		Hydrolysis- isolate	NH	NH	NH	SH	NH
10	*E. coli* FS	Disc diffusion	S	S	S	S	S
		Hydrolysis- urine	SH	NH	NH	NR	NH
		Hydrolysis- isolate	SH	NH	SH	NH	NH
11	*E. coli* ESBL	Disc diffusion	R (16)	R	R	R (13)	R
		Hydrolysis- urine	H	NRR	H	NR	H
		Hydrolysis- isolate	H	H	H	H	H
12	*E. coli* ESBL	Disc diffusion	R	R	R	R	R
		Hydrolysis- urine	H	H	H	H	H
		Hydrolysis- isolate	H	H	H	H	H
13	*E. coli* ESBL	Disc diffusion	R	R	R	R (12)	R
		Hydrolysis- urine	H	H	H	NH	H
		Hydrolysis- isolate	H	H	H	H	H

No	Species & Mechanism	Methods	FEP	CRO	CPD	CAZ	CTX
14	*E. coli* ESBL	Disc diffusion	R (13)	R	R	R (16)	R
		Hydrolysis- urine	H	H	H	NH	H
		Hydrolysis- isolate	H	H	H	H	H
15	*E. coli* Ceph-Res but no ESBL/AmpC[1]	Disc diffusion	R (19)	R	R	R (12)	R
		Hydrolysis- urine	H	H	H	NH	H
		Hydrolysis- isolate	H	H	H	NH	H
16	*E. coli* FS	Disc diffusion	S	S	S	S	S
		Hydrolysis- urine	NH	NH	NH	NH	NH
		Hydrolysis- isolate	NH	NH	NH	NH	NH
17	*E. coli* ESBL	Disc diffusion	R	R	R	R (12)	R
		Hydrolysis- urine	H	H	H	NH	H
		Hydrolysis- isolate	H	H	H	H	H
18	*E. coli* ESBL	Disc diffusion	R	R	R	R	R
		Hydrolysis- urine	H	H	H	H	H
		Hydrolysis- isolate	H	H	H	H	H
19	*E. coli* FS	Disc diffusion	S	S	S	S	S
		Hydrolysis- urine	NH	NH	NH	NH	SH
		Hydrolysis- isolate	NH	NH	NH	NH	NH
20	*E. coli* FS	Disc diffusion	S	S	S	S	S
		Hydrolysis- urine	NH	NH	NH	NH	SH
		Hydrolysis- isolate	NH	NH	NH	NH	NH
21	*E. coli* ESBL	Disc diffusion	R	R	R	R (11)	R
		Hydrolysis- urine	H	H	H	H	H
		Hydrolysis- isolate	NH	H	H	H	NRR
22	*E. coli* FS	Disc diffusion	S	S	S	S	S
		Hydrolysis- urine	NH	NH	NH	NH	NH
		Hydrolysis- isolate	NH	NH	NH	NH	NH
23	*E. coli* ESBL	Disc diffusion	R (15)	R	R	R (11)	R
		Hydrolysis- urine	H	H	H	NH	H
		Hydrolysis- isolate	H	H	H	H	NH
24	*E. coli* ESBL	Disc diffusion	R (15)	R	R	R (10)	R
		Hydrolysis- urine	H	H	H	NH	H
		Hydrolysis- isolate	H	H	H	H	H
25	*E. coli* FS	Disc diffusion	S	S	S	S	S
		Hydrolysis- urine	NH	NH	NH	NH	NH
		Hydrolysis- isolate	NH	NH	NH	NH	NH
26	*E. coli* ESBL	Disc diffusion	R (13)	R	R	R	R
		Hydrolysis- urine	H	H	H	H	H
		Hydrolysis- isolate	H	H	H	H	NRR
27	*E. coli* ESBL	Disc diffusion	R	R	R	R	R
		Hydrolysis- urine	H	H	NH	NH	SH
		Hydrolysis- isolate	H	H	H	H	H
28	*E. coli* ESBL	Disc diffusion	R	R	R	R	R
		Hydrolysis- urine	H	H	H	H	H
		Hydrolysis- isolate	H	H	H	H	H

No	Species & Mechanism	Methods	FEP	CRO	CPD	CAZ	CTX
29	*E. coli* ESBL	Disc diffusion	R (13)	R	R	R	R
		Hydrolysis- urine	H	H	H	H	H
		Hydrolysis- isolate	H	H	H	H	H
30	*E. coli* FS	Disc diffusion	S	S	S	S	S
		Hydrolysis- urine	NH	NH	NH	NH	NH
		Hydrolysis- isolate	NH	NH	NH	NH	NH
31	*E. coli* ESBL	Disc diffusion	R	R	R	R (13)	R
		Hydrolysis- urine	H	H	H	H	H
		Hydrolysis- isolate	H	H	H	H	H
32	*E. coli* ESBL	Disc diffusion	R	R	R	R (14)	R
		Hydrolysis- urine	H	H	H	H	H
		Hydrolysis- isolate	H	NH	H	NH	H
33	*E. coli* ESBL	Disc diffusion	R	R	R	R	R
		Hydrolysis- urine	SH	H	H	H	H
		Hydrolysis- isolate	H	H	H	H	H
34	*E. coli* ESBL	Disc diffusion	R	R	R	R	R
		Hydrolysis- urine	H	H	H	H	H
		Hydrolysis- isolate	H	H	H	H	H
35	*E. coli* ESBL	Disc diffusion	R	R	R	R	R
		Hydrolysis- urine	H	H	H	H	H
		Hydrolysis- isolate	H	H	H	H	H
36	*E. coli* ESBL	Disc diffusion	R (15)	R	R	R (10)	R
		Hydrolysis- urine	H	H	H	H	H
		Hydrolysis- isolate	H	H	H	H	H
37	*E. coli* ESBL	Disc diffusion	R	R	R	R (12)	R
		Hydrolysis- urine	NRR	H	H	H	H
		Hydrolysis- isolate	H	H	H	H	H
38	*E. coli* ESBL	Disc diffusion	R	R	R	R (12)	R
		Hydrolysis- urine	H	H	H	H	H
		Hydrolysis- isolate	H	H	SH	H	H
39	*E. coli* ESBL	Disc diffusion	R	R	R	R (20)	R
		Hydrolysis- urine	SH	NH	NH	NH	NRR
		Hydrolysis- isolate	H	H	H	H	H
40	*E. coli* FS	Disc diffusion	S	S	S	S	S
		Hydrolysis- urine	NH	NH	NH	NH	NRR
		Hydrolysis- isolate	NH	NH	NH	NH	NH
41	*E. coli* FS	Disc diffusion	S	S	S	S	S
		Hydrolysis- urine	NH	NH	NH	NH	NH
		Hydrolysis- isolate	NH	NH	NH	NH	NH
42	*E. coli* FS	Disc diffusion	S	S	S	S	S
		Hydrolysis- urine	NH	NH	NH	NH	NH
		Hydrolysis- isolate	NH	NH	NRR	NH	NH
43	*E. coli* FS	Disc diffusion	S	S	S	S	S
		Hydrolysis- urine	NH	NH	NH	SH	NH
		Hydrolysis- isolate	NH	NH	NH	NH	NH

No	Species & Mechanism	Methods	FEP	CRO	CPD	CAZ	CTX
44	*E. coli* ESBL	Disc diffusion	R	R	R	R	R
		Hydrolysis- urine	H	H	H	H	H
		Hydrolysis- isolate	H	H	H	H	H
45	*E. coli* ESBL	Disc diffusion	R	R	R	R (14)	R
		Hydrolysis- urine	H	H	H	H	H
		Hydrolysis- isolate	H	H	H	H	H
46	*E. coli* Ceph-Res but no ESBL/AmpC[1]	Disc diffusion	R	R	R	R	R
		Hydrolysis- urine	H	H	H	H	H
		Hydrolysis- isolate	H	H	H	H	H
47	*K. pneumoniae* ESBL	Disc diffusion	R (15)	R	R	R (10)	R
		Hydrolysis- urine	H	H	H	H	H
		Hydrolysis- isolate	H	H	H	H	H
48	*E. coli* FS	Disc diffusion	S	S	S	S	S
		Hydrolysis- urine	NH	NH	NH	NH	NH
		Hydrolysis- isolate	NH	NH	NH	NH	NH
49	*K. pneumoniae* ESBL	Disc diffusion	R (15)	R	R	R (11)	R
		Hydrolysis- urine	H	H	H	H	H
		Hydrolysis- isolate	H	H	H	H	H
50	*E. coli* ESBL	Disc diffusion	R (15)	R	R	R (18)	R
		Hydrolysis- urine	H	H	H	NH	H
		Hydrolysis- isolate	H	H	H	H	H
51	*E. coli* ESBL	Disc diffusion	R	R	R	R	R
		Hydrolysis- urine	H	H	H	SH	H
		Hydrolysis- isolate	H	H	H	H	H
52	*E. coli* Ceph-Res but no ESBL/AmpC[1]	Disc diffusion	R (14)	R	R	R (20)	R
		Hydrolysis- urine	H	H	H	H	H
		Hydrolysis- isolate	H	H	H	H	H
53	*E. coli* FS	Disc diffusion	S	S	S	S	S
		Hydrolysis- urine	NH	NH	NH	NH	NH
		Hydrolysis- isolate	NRR	NH	NH	NH	NH
54	*E. coli* FS	Disc diffusion	S	S	S	S	S
		Hydrolysis- urine	NH	NH	NH	NH	NH
		Hydrolysis- isolate	NRR	NH	NH	NH	NH
55	*E. coli* ESBL	Disc diffusion	R	R	R	R (12)	R
		Hydrolysis- urine	H	H	H	H	H
		Hydrolysis- isolate	H	H	H	H	H
56	*E. coli* ESBL	Disc diffusion	R (15)	R	R	R	R
		Hydrolysis- urine	H	H	H	H	H
		Hydrolysis- isolate	H	H	H	H	H
57	*E. coli* ESBL	Disc diffusion	R (17)	R	R	R	R
		Hydrolysis- urine	H	H	H	H	H
		Hydrolysis- isolate	H	H	H	H	H
58	*E. coli* ESBL	Disc diffusion	R (19)	R	R	R (18)	R
		Hydrolysis- urine	NRR	SH	SH	NH	SH
		Hydrolysis- isolate	H	H	H	H	H

No	Species & Mechanism	Methods	FEP	CRO	CPD	CAZ	CTX
59	*E. coli* ESBL	Disc diffusion	R (18)	R	R	R (19)	R
		Hydrolysis- urine	H	H	H	H	H
		Hydrolysis- isolate	H	H	H	H	H
60	*E. coli* ESBL	Disc diffusion	R (18)	R	R	R (10)	R
		Hydrolysis- urine	H	H	H	H	H
		Hydrolysis- isolate	H	H	H	H	H
61	*E. coli* ESBL	Disc diffusion	R (20)	R	R	R	R
		Hydrolysis- urine	H	H	H	SH	H
		Hydrolysis- isolate	H	H	H	H	H
62	*E. coli* FS	Disc diffusion	S	S	S	S	S
		Hydrolysis- urine	NH	NH	NH	NH	NH
		Hydrolysis- isolate	NH	NH	NH	NH	NH
63	*E. coli* FS	Disc diffusion	S	S	S	S	S
		Hydrolysis- urine	NH	NH	NH	NH	NH
		Hydrolysis- isolate	NH	NH	NH	NH	NH
64	*C. frundii* AmpC	Disc diffusion	S	S	R	S	S
		Hydrolysis- urine	NH	NH	NH	NH	NH
		Hydrolysis- isolate	NH	NH	NH	NH	NH
65	*E. cloacae* AmpC	Disc diffusion	R (11)	R	R	R	R
		Hydrolysis- urine	NH	NH	SH	NH	NH
		Hydrolysis- isolate	NH	NH	H	H	H
66	*E. coli* ESBL	Disc diffusion	R	R	R	R	R
		Hydrolysis- urine	H	H	H	H	H
		Hydrolysis- isolate	H	H	H	H	H
67	*E. coli* AmpC	Disc diffusion	S	S	R	S	S
		Hydrolysis- urine	SH	NH	SH	SH	NH
		Hydrolysis- isolate	NH	NH	NH	NH	NH
68	*E. coli* ESBL	Disc diffusion	R	R	R	R	R
		Hydrolysis- urine	NH	NH	SH	NH	SH
		Hydrolysis- isolate	H	H	H	H	H
69	*E. coli* ESBL	Disc diffusion	R (21)	R	R	R (21)	R
		Hydrolysis- urine	H	H	H	NH	H
		Hydrolysis- isolate	H	H	H	H	H
70	*C. braaki* AmpC	Disc diffusion	S	R	R	R	R
		Hydrolysis- urine	NH	NH	NRR	NH	NH
		Hydrolysis- isolate	SH	NH	H	H	H
71	*E. coli* FS	Disc diffusion	S	S	S	S	S
		Hydrolysis- urine	NH	NH	NH	NH	NH
		Hydrolysis- isolate	NH	NH	NH	NH	NH
72	*E. coli* FS	Disc diffusion	S	S	S	S	S
		Hydrolysis- urine	NH	NH	NH	NH	NH
		Hydrolysis- isolate	NH	NH	NH	NH	NH
73	*S. marcescens* AmpC	Disc diffusion	S	S	R	S	S
		Hydrolysis- urine	SH	NH	H	NH	SH
		Hydrolysis- isolate	NH	NH	H	NH	SH

No	Species & Mechanism	Methods	FEP	CRO	CPD	CAZ	CTX
74	*K. pneumonia* AmpC	Disc diffusion	S	I (24)	R (14)	S	I (24)
		Hydrolysis- urine	NH	NH	NH	NH	SH
		Hydrolysis- isolate	SH	SH	NH	NH	SH
75	*E. coli* AmpC	Disc diffusion	I (28)	I (25)	R (11)	R (21)	R (22)
		Hydrolysis- urine	H	NH	SH	NH	SH
		Hydrolysis- isolate	H	H	SH	H	SH
76	*E. coli* AmpC	Disc diffusion	S (30)	I (24)	R	R (20)	R (20)
		Hydrolysis- urine	NH	NH	SH	NH	NH
		Hydrolysis- isolate	SH	NH	H	SH	NH
77	*E. coli* AmpC	Disc diffusion	S (30)	S (28)	R	R (19)	R (20)
		Hydrolysis- urine	NH	NH	NH	NH	NH
		Hydrolysis- isolate	SH	NH	H	NH	NH
78	*E. coli* AmpC	Disc diffusion	R (24)	R	R	R	R
		Hydrolysis- urine	SH	NH	H	SH	SH
		Hydrolysis- isolate	H	SH	H	H	NRR
79	*E. coli* FS	Disc diffusion	S	S	S	S	S
		Hydrolysis- urine	NH	NH	NH	NH	NH
		Hydrolysis- isolate	NH	NH	NH	NH	NH
80	*E. aerogenes* AmpC	Disc diffusion	S	R (20)	R	R (19)	R (18)
		Hydrolysis- urine	NH	NH	NH	NH	NH
		Hydrolysis- isolate	SH	H	H	H	H
81	*E. coli* AmpC	Disc diffusion	S (30)	S (30)	R (11)	R (20)	I (24)
		Hydrolysis- urine	SH	NR	NH	NH	SH
		Hydrolysis- isolate	SH	NH	H	H	SH
82	*E. coli* AmpC	Disc diffusion	S (30)	I (25)	R	R (17)	R (22)
		Hydrolysis- urine	NH	NH	NH	NH	NH
		Hydrolysis- isolate	H	NH	H	NH	H
83	*E. coli* AmpC	Disc diffusion	S (30)	I (25)	R	R (17)	R (21)
		Hydrolysis- urine	NH	NH	NH	NH	NH
		Hydrolysis- isolate	H	NH	H	NH	H
84	*E. coli* AmpC	Disc diffusion	S (30)	I (24)	R	R (11)	R (21)
		Hydrolysis- urine	NH	NR	NR	NH	NH
		Hydrolysis- isolate	H	NH	H	SH	H
85	*E. coli* AmpC	Disc diffusion	R (20)	R	R	R	R
		Hydrolysis- urine	NH	NH	H	NH	NH
		Hydrolysis- isolate	H	NH	H	H	NH
86	*E. aerogenes* AmpC	Disc diffusion	R (25)	R (12)	R	R	R
		Hydrolysis- urine	NH	NH	NH	NH	NH
		Hydrolysis- isolate	H	H	H	H	H
87	*E. cloacae* AmpC	Disc diffusion	S	R	R	R	R
		Hydrolysis- urine	NH	NH	H	NH	SH
		Hydrolysis- isolate	H	NH	H	NH	NH
88	*E. aerogenes* AmpC	Disc diffusion	S (30)	R (17)	R	R (16)	R (17)
		Hydrolysis- urine	SH	NH	SH	NH	NH
		Hydrolysis- isolate	NH	NH	SH	NH	NH

No	Species & Mechanism	Methods	FEP	CRO	CPD	CAZ	CTX
89	*E. coli* AmpC	Disc diffusion	R (18)	R	R	R	R
		Hydrolysis- urine	SH	H	H	NH	NH
		Hydrolysis- isolate	H	NH	SH	NH	NH
90	*E. aerogenes* AmpC	Disc diffusion	R (25)	R	R	R	R
		Hydrolysis- urine	SH	NH	H	NH	SH
		Hydrolysis- isolate	H	SH	SH	NH	NRR
91	*E. coli* AmpC	Disc diffusion	R (26)	R	R	R (14)	R (14)
		Hydrolysis- urine	SH	NH	SH	NH	SH
		Hydrolysis- isolate	H	SH	SH	NH	SH

Legend: R- resistant; S- susceptible; H- hydrolysed; NH- no hydrolysed; SH-slow hydrolysis; NR- no result; NRR-no reliable result; - agreement between hydrolysis using urine and isolate *vs*. phenotypic results; - disagreement between hydrolysis using urine and isolate *vs*. phenotypic results; - disagreement between hydrolysis using urine and isolate. [1] Cephalosporin resistance and ESBL/AmpC producers were detected using a phenotypic test (D68C ESBL & AmpC Detection Discs Set).

Table 27. Detection of cephalosporin hydrolysis by MALDI-TOF for all urines tested directly.

Urine (n=91)	MALDI-TOF	BSAC Disc Diffusion Results				
		R (50)		I (1)	S (40)	Total
		no zone (22)	zone (28)			
Cefepime	Hydrolysis detected	18	20	1	-	39
	No hydrolysis detected	1	3	-	35	39
	Slow hydrolysis*	2	4	-	5	11
	No reliable result**	1[1]	1[2]	-	-	2
	Total	22	28	1	40	91
	Sensitivity	91.7%[4]		91.8%[3]		
	Specificity	85.4%[4]		87.5%[3]		
		R (54)		I (6)	S (31)	Total
		no zone (50)	zone (4)			
Ceftriaxone	Hydrolysis detected	39	1	-	-	40
	No hydrolysis detected	9	3	5	30	47
	Slow hydrolysis*	1	-	-	-	1
	No reliable result**	1[1]	-	-	-	1
	No result	-	-	1	1	2
	Total	50	4	6	31	91
	Sensitivity	77.4%[4]		70.7%[3]		
	Specificity	100%				

Urine (n=91)	MALDI-TOF	BSAC Disc Diffusion Results				
		R (65)		I (0)	S (26)	Total
		no zone (62)	zone (3)			
Cefpodoxime	Hydrolysis detected	45	-	-	-	45
	No hydrolysis detected	8	2	-	25	35
	Slow hydrolysis*	8	1	-	-	9
	No result	1	-	-	1	2
	Total	62	3	-	26	91
	Sensitivity	84.4%				
	Specificity	100%				
		R (61)		I (0)	S(30)	Total
		no zone (26)	zone (35)			
Ceftazidime	Hydrolysis detected	14	13	-	-	27
	No hydrolysis detected	9	21	-	21	51
	Slow hydrolysis*	2	0	-	2	4
	No result	1	1	-	7	9
	Total	26	35	-	30	91
	Sensitivity	49.2%				
	Specificity	91.3%				
		R (60)		I (2)	S (29)	Total
		no zone (50)	zone (10)			
Cefotaxime	Hydrolysis detected	38	1	-	-	39
	No hydrolysis detected	5	7	-	25	37
	Slow hydrolysis*	6	2	2	3	13
	No reliable result**	1[2]	-	-	1[2]	2
	Total	50	10	2	29	91
	Sensitivity	79.7%[4]			80.3%[3]	
	Specificity	83.3%[4]			89.3%[3]	

Legend: R- resistant; no zone- high level resistance; zone- low-level resistance with zones in a range 10-22 mm; S- susceptible, I- intermediate; *Slow hydrolysis- specimens with intensity distribution between not hydrolysed and hydrolysed, **No reliable results- discrepancy between categorisation from automated software (MBT-STAR-BL or its prototype) and raw peak profile provided by FlexAnalysis software ([1]not hydrolysed *vs*. cephalosporin peak invisible, [2]hydrolysed *vs*. cephalosporin peak visible). Sensitivity and specificity were calculated using the online clinical calculator (see Tables in Appendix B).

[3]specimens with intermediate zones classified as resistant
[4]specimens with intermediate zones classified as suseptible

Table 28. Detection of cephalosporin hydrolysis by MALDI-TOF for isolates cultivated from urines.

Cultivated Isolate (n=91)	MALDI-TOF	BSAC Disc Diffusion Results				
		R (50)		I (1)	S (40)	Totals
		no zone (22)	zone (28)			
Cefepime	Hydrolysis detected	21	28	1	4	54
	No hydrolysis detected	1	-	-	27	28
	Slow hydrolysis*	-	-	-	7	7
	No reliable result**	-	-	-	2[2]	2
	Total	22	28	1	40	91
	Sensitivity	98%				
	Specificity	69.2%[4]		71.1%[3]		
		R (54)		I (6)	S (31)	Total
		no zone (50)	zone (4)			
Ceftriaxone	Hydrolysis detected	41	3	1	-	45
	No hydrolysis detected	5	1	4	31	41
	Slow hydrolysis*	4	-	1	-	5
	Total	50	4	5	31	91
	Sensitivity	88.9%[4]		83.3%[3]		
	Specificity	94.6%[4]		100%[3]		
		R (65)		I (0)	S (26)	Total
		no zone (62)	zone (3)			
Cefpodoxime	Hydrolysis detected	55	1	-	-	56
	No hydrolysis detected	2	1	-	24	27
	Slow hydrolysis*	5	1	-	1	7
	No reliable result**	-	-	-	1[1]	1
	Total	62	3	-	26	91
	Sensitivity	95.4%				
	Specificity	96%				
Ceftazidime		R (61)		I (0)	S (30)	Total
		no zone (26)	zone (35)			
	Hydrolysis detected	22	25	-	-	47
	No hydrolysis detected	3	8	-	29	40
	Slow hydrolysis*	1	2	-	1	4
	Total	26	35	-	30	91
	Sensitivity	82%				
	Specificity	96.7%				

Cultivated Isolate (n=91)	MALDI-TOF	BSAC Disc Diffusion Results				
		R (60)		I (2)	S (29)	Total
		no zone (50)	zone (10)			
Cefotaxime	Hydrolysis deteced	42	5	-	-	47
	No hydrolysis detected	4	3	-	28	35
	Slow hydrolysis*	-	2	2	1	5
	No reliable result**	4[1,2]	-	-	-	4
	Total	50	10	2	29	91
	Sensitivity	87.5%[4]		87.9%[3]		
	Specificity	90.3%[4]		96.7%[3]		

Abbreviations and features as for Table 27.

Table 29. Cephalosporin-hydrolysis for ESBL producers from urines and the bacterial isolates cultivated from them.

ESBL (n=43)	MALDI-TOF	Urine		Cultivated isolates	
		BSAC Disc Diffusion Method			
		R (43)		R (43)	
		no zone (22)	zone (21)	no zone (22)	zone (21)
Cefepime	Hydrolysis detected	18	20	21	21
	No hydrolysis detected	1	-	1	-
	Slow hydrolysis*	2	-	-	-
	No reliable result**	1[1]	1[2]	-	-
	Total	22	21	22	21
Ceftriaxone		**no zone (42)**	**zone (1)**	**no zone (42)**	**zone (1)**
	Hydrolysis detected	38	1	41	1
	No hydrolysis detected	2	-	1	-
	Slow hydrolysis*	1	-	-	-
	No reliable result**	1[1]	-	-	-
	Total	42	1	42	1
Cefpodoxime		**no zone (43)**	**zone (0)**	**no zone (43)**	**zone (0)**
	Hydrolysis detected	39	-	42	-
	No hydrolysis detected	2	-	-	-
	Slow hydrolysis*	2	-	1	-
	Total	43	-	43	-
Ceftazidime		**no zone (18)**	**zone (25)**	**no zone (18)**	**zone (25)**
	Hydrolysis detected	14	13	17	22
	No hydrolysis detected	2	11	-	3
	Slow hydrolysis*	1	-	1	-
	No result	1	1	-	-
	Total	18	25	18	25
Cefotaxime		**no zone (42)**	**zone (1)**	**no zone (42)**	**zone (1)**
	Hydrolysis detected	38	1	40	-
	No hydrolysis detected	-	-	-	1
	Slow hydrolysis*	3	-	-	-
	No reliable result**	1[2]	-	2[1]	-
	Total	42	1	42	1

ESBL assay	**Urines**	**Isolates**
Sensitivity	91.4%	98.1%

Abbreviations and features as for Table 27.

Table 30. Cephalosporin hydrolysis for AmpC β-lactamase producers from urines and bacterial isolates cultivated from them.

AmpC (n=22)	MALDI-TOF	Urine			Cultivated isolates		
		BSAC Disc Diffusion Method					
		R (7)		S/I (15)	R (7)		S/I (15)
		no zone (0)	zone (7)		no zone (0)	zone (7)	
Cefepime	Hydrolysis detected	-	-	1	-	7	5
	No hydrolysis detected	-	3	10	-	-	4
	Slow hydrolysis*	-	4	4	-	-	6
	Total	-	7	15	-	7	15
Ceftriaxone		R (11)		S/I (11)	R (11)		S/I (11)
		no zone (8)	zone (3)		no zone (8)	zone (3)	
	Hydrolysis detected	1	-	-	-	2	1
	No hydrolysis detected	7	3	9	4	1	9
	Slow hydrolysis*	-	-	-	4	-	1
	No result	-	-	2	-	-	-
	Total	8	3	11	8	3	11
Cefpodoxime		R (22)		S/I (0)	R (22)		S/I (0)
		no zone (19)	zone (3)		no zone (19)	zone (3)	
	Hydrolysis detected	6	-	-	13	1	-
	No hydrolysis detected	6	2	-	2	1	-
	Slow hydrolysis*	6	1	-	4	1	-
	No result	1	-	-	-	-	-
	Total	19	3	-	19	3	-
Ceftazidime		R (18)		S/I (4)	R (18)		S/I (4)
		no zone (8)	zone (10)		no zone (8)	zone (10)	
	Hydrolysis detected	-	-	-	5	3	-
	No hydrolysis detected	7	10	3	3	5	4
	Slow hydrolysis*	1	-	1	-	2	-
	No result	-	-	-	-	-	-
	Total	8	10	4	8	10	4

AmpC (n=22)	MALDI-TOF	Urine			Cultivated isolates		
		BSAC Disc Diffusion Method					
Cefotaxime		R (17)		S/I (5)	R (17)		S/I (5)
		no zone (8)	zone (9)		no zone (8)	zone (9)	
	Hydrolysis detected	-	-	-	3	4	-
	No hydrolysis detected	5	7	2	3	3	2
	Slow hydrolysis*	3	2	3	-	2	3
	No reliable result**	-	-	-	2	-	-
	Total	8	9	5	8	9	5
AmpC assay		Urines			Isolates		
Sensitivity		32.4%			69.7%		

Abbreviations and features as for Table 27.

3.2.2.4 Cephalosporin hydrolysis by MALDI-TOF versus ESBL and AmpC gene detection by sequencing

The main purpose of this study was to compare β-lactamase gene profiles found by sequencing with cephalosporin hydrolysis assays using MALDI-TOF. For this purpose 51 out of the 91 bacterial isolates used in MALDI-TOF hydrolysis assays were sequenced to identify β-lactamase genes. These 51 isolates were selected on the basis of phenotypic resistance profiles. These included 41 ESBL producers (38 *E. coli* and 3 *K. pneumoniae*) and 10 AmpC producers comprising *E. coli* (n=4), *E. aerogenes* (n=3), *E. cloacae* (n=2) and *C. braakii* (n=1) (see Table 23). The full β-lactamase profiles for all these sequenced isolates are presented in Table 33.

Putative ESBL producers

ESBL genes were found in 38/41 isolates with ESBL phenotypes (see Table 31 and 33). These mostly comprised: $bla_{\text{CTX-M}}$ types, specifically: 2 $bla_{\text{CTX-M -3,}}$ 4 $bla_{\text{CTX-M -9,}}$ 2 $bla_{\text{CTX-M -14,}}$ 26 $bla_{\text{CTX-M -15}}$ and 4 $bla_{\text{CTX-M -27.}}$ In addition, $bla_{\text{SHV-27}}$ was identified in the three *K. pneumoniae* isolates. Hydrolysis of all five tested cephalosporins was detected for 32 out of these 38 isolates with $bla_{\text{CTX-M}}$ genes, whilst one *E. coli* with $bla_{\text{CTX-M -15}}$ failed to hydrolyse ceftazidime and another failed to hydrolyse cefotaxime; two further *E. coli* with ESBLs, one with $bla_{\text{CTX-M -15}}$ did not showed hydrolysis for cefepime and cefotaxime and one with $bla_{\text{CTX-M -27}}$ failed to hydrolyse ceftriaxone and ceftazidime. Slow hydrolysis was seen for one *E. coli* with $bla_{\text{CTX-M -15}}$ for ceftazidime only. All *K. pneumoniae* isolates with $bla_{\text{SHV-27}}$ together with $bla_{\text{CTX-M-15}}$ showed hydrolysis for all cephalosporins tested (Table 24, 25, 31 and 33).

Hydrolysis of 4 or 5 cephalosporins tested was noted for the 3 isolates identified phenotypically as ESBL producers but no ESBL gene was found (see Table 31 and 33). These 3 isolates probably lost ESBL during storage, after the MALDI-TOF assay but before sequencing.

Table 31. Number of cephalosporins hydrolysed for isolates with ESBLs phenotypes and ESBLs confirmed by sequencing.

	ESBL genes present						ESBL phenotype but not confirmed by sequncing
No of cephalosporins with hydrolysis detected	*bla*$_{CTX-M-3}$ group 1 (n=2)	*bla*$_{CTX-M-15}$ group 1 (n=26)	*bla*$_{CTX-M-27}$ group 9 (n=4)	*bla*$_{CTX-M-9}$ group 9 (n=4)	*bla*$_{CTX-M-14}$ group 9 (n=2)	*bla*$_{CTX-M-15}$ plus *bla*$_{SHV-27}$	
0	-	-	-	-	-	-	-
1	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-
3	-	1[b]	1[c]	-	-	-	-
4	-	2[a]	-	-	-	-	1
5	2	23	3	4	2	3	2

Legend: [a] Hydrolysis was not detected for cefepime or cefotaxime (one isolate each); [b] hydrolysis was not detected for cefepime and cefotaxime, [c] hydrolysis was not detected for ceftriaxone and ceftazidime.

Putative AmpC producers

Seven isolates, including four *E. coli* (no 44, 45, 49, 51), two *Enterobacter* spp. (no 40, 50) and one *Citrobacter braakii* (no 43) with phenotypes suggesting hyperproduced AmpC were sequenced (see Table 33). These isolates were resistant to cefoxitin and cefpodoxime, with much greater resistance to ceftazidime, cefotaxime and ceftriaxone than for cefepime. We also included two *Enterobacter* spp. (no 47 and 48) resistant only to cefpodoxime and cefoxitin and *Klebsiella oxytoca* (no 46) which was initially resistant to all cephalosporins tested (Table 33). *Enterobacter* spp. (no 47 and 48) initially tested more widely resistant to cephalosporins by disc diffusion suggesting AmpC derepressed mutants, probably contained subpopulations, perhaps later overgrown, of AmpC inducible strain. Table 32 shows the cephalosporin hydrolysis for sequenced AmpC β-lactamase producers.

Sequencing detected plasmid-mediated *ampC* genes $bla_{\text{CMY-42}}$ (no 44), $bla_{\text{CMY-33}}$ (no 45), $bla_{\text{CMY-2}}$ (no 51) and $bla_{\text{CMY-44}}$ (no 49) in the four *E. coli*. Two of these (no 44, 45) hydrolysed ceftazidime; all hydrolysed cefpodoxime, although two showed slow hydrolysis (no 49, 51), two (strains no 44, 51) showed slow hydrolysis for ceftriaxone and only one (strain no 51) slow hydrolysed cefotaxime. Surprisingly all 4 isolates hydrolysed cefepime.

In the case of *E. cloacae* (no 40) sequencing flagged $bla_{\text{ACT-14,}}$ which can be plasmid mediated *ampC* but is more likely chromosomal variant previously found in *Enterobacter* avian isolates (Literak *et al.*, 2014).

In the remaining isolates (*E. cloacae,* two *E. aerogenes* and *C. braaki*) sequencing was not prove helpful, as what matters in the expression of chromosomal AmpC rather than it is presence. The reference database was designed to seek mutations rather than cause chromosomal upregulation of *ampC* genes.

Curiously, sequencing detected $bla_{\text{OXY-2}}$ gene in isolate (no 46) suggesting it was *K. oxytoca* though MALDI-TOF identified *E. aerogenes* and sequencing data indicated 25% identity to *E. aerogenes* reference genome suggesting the presence of heterogenous population.

Table 32. Number of cephalosporins hydrolysed for sequenced isolates producing AmpC β-lactamases.

	AmpC producers		
No of cephalosporins with hydrolysis detected*	*E.coli* plasmid AmpC Isolates no 44, 45, 49, 51 (n=4)	*E. cloacae/ E. aerogenes/C. brakkii* derepressed AmpC Isolates nr: 40, 43, 50 (n=3)	Heterogenous population Isolates nr: 46, 47, 48 (n=3)
0	-	-	-
1	-	-	1
2	1	-	1
3	1	1	-
4	2	1	-
5	-	1	1

*Table contains data for cephalosporins with full and slow hydrolysis.

Table 33. Cephalosporin hydrolysis results versus phenotypic and sequence profile for all sequenced bacterial isolates.

Strain No.	ID	Mechanism	Methods	Penicillins			Cephalosporin							
				AMO	AUG	TZP	CAZ	CTX	CPD	CRO	FEP	CL	CXM	FOX
1	*E. coli*	ESBL	Disc Diffusion	R	S	S	R	R	R	R	R (14)	R	R	-
			Hydrolysis	-			H	H	H	H	H	-	-	-
			Sequencing	-			CTX-M-15							
2	*E. coli*	ESBL	Disc Diffusion	R	R	S	R (19)	R (14)	R	R (15)	R (23)	R	R	-
			Hydrolysis	-			NH	H	H	H	H	-	-	-
			Sequencing	OXA-1			CTX-M-15							
3	*E. coli*	ESBL	Disc Diffusion	R	S	S	R (13)	R	R	R	R (16)	R	R	-
			Hydrolysis	-			H	H	H	H	H	-	-	-
			Sequencing	-			CTX-M-27 (gr 9)							
4	*E. coli*	ESBL	Disc Diffusion	R	R	S	R (12)	R	R	R	R	R	R	-
			Hydrolysis	-			H	H	H	H	H	-	-	-
			Sequencing	OXA-1			CTX-M-15							
5	*E. coli*	ESBL	Disc Diffusion	R	R	S	R	R	R	R	R	R	R	-
			Hydrolysis	-			H	H	H	H	H	-	-	-
			Sequencing	OXA-1			CTX-M-15							
6	*E. coli*	ESBL	Disc Diffusion	R	R	S	R (12)	R	R	R	R	R	R	-
			Hydrolysis	-			H	H	H	H	H	-	-	-
			Sequencing	TEM-33 (2br-inhibitor resistant)			CTX-M-9							

Strain No.	ID	Mechanism	Methods	Penicillins			Cephalosporin							
				AMO	AUG	TZP	CAZ	CTX	CPD	CRO	FEP	CL	CXM	FOX
7	*E. coli*	ESBL	Disc Diffusion	R	S	S	R (16)	R	R	R	R (13)	R	R	-
			Hydrolysis	-			H	H	H	H	H	-	-	-
			Sequencing	TEM-198*			CTX-M-14							
8	*E. coli*	Ceph-Res but no ESBL/ AmpC	Disc Diffusion	R	S	S	R (12)	R	R	R	R (19)	R	R	-
			Hydrolysis	-			NH	H	H	H	H	-	-	-
			Sequencing	TEM-186 (2b-broad spectrum)			-							
9	*E. coli*	ESBL	Disc Diffusion	R	R	S	R	R	R	R	R	R	R	-
			Hydrolysis	-			H	H	H	H	H	-	-	-
			Sequencing	OXA-1			CTX-M-15							
10	*E. coli*	ESBL	Disc Diffusion	R	R	S	R (11)	R	R	R	R	R	R	-
			Hydrolysis	-			H	NRR	H	H	NH	-	-	-
			Sequencing	OXA-1			CTX-M-15							
11	*E. coli*	ESBL	Disc Diffusion	R	R	S	R (10)	R	R	R	R (15)	R	R	-
			Hydrolysis	-			H	H	H	H	H	-	-	-
			Sequencing	OXA-1			CTX-M-15							
12	*E. coli*	ESBL	Disc Diffusion	R	R	S	R (11)	R	R	R	R (15)	R	R	-
			Hydrolysis	--			H	NH	H	H	H	-	-	-
			Sequencing	OXA-1			CTX-M-15							
13	*E. coli*	ESBL	Disc Diffusion	R	S	S	R	R	R	R	R (13)	R	R	-
			Hydrolysis	-			H	NRR	H	H	H	-	-	-
			Sequencing	-			CTX-M-15							
14	*E. coli*	ESBL	Disc Diffusion	R	S	S	R	R	R	R	R	R	R	-
			Hydrolysis	-			H	H	H	H	H	-	-	-
			Sequencing	TEM-34 (2br-inhibitor resistant)			CTX-M-15							

Strain No.	ID	Mechanism	Methods	Penicillins			Cephalosporin							
				AMO	AUG	TZP	CAZ	CTX	CPD	CRO	FEP	CL	CXM	FOX
15	*E. coli*	ESBL	Disc Diffusion	R	R	S	R	R	R	R	R	R	R	-
			Hydrolysis				H	H	H	H	H	-	-	-
			Sequencing	TEM-198*, OXA-1			CTX-M-15							
16	*K. pneumoniae*	ESBL	Disc Diffusion	R	R	S	R	R	R	R	R (13)	R	R	-
			Hydrolysis				H	H	H	H	H	-	-	-
			Sequencing	TEM-198*, OXA-1			CTX-M-15, SHV-27 (2be- extended spectrum)							
17	*E. coli*	ESBL	Disc Diffusion	R	S	S	R (14)	R	R	R	R	R	R	-
			Hydrolysis	-			NH	H	H	NH	H	-	-	-
			Sequencing	TEM-198*			CTX-M-27 (gr 9)							
18	*E. coli*	ESBL	Disc Diffusion	R	S	S	R (10)	R	R	R	R (15)	R	R	-
			Hydrolysis	-			H	H	H	H	H	-	-	-
			Sequencing	-			CTX-M-27 (gr-9)							
19	*E. coli*	ESBL	Disc Diffusion	R	R	S	R	R	R	R	R	R	R	-
			Hydrolysis	-			H	H	H	H	H	-	-	-
			Sequencing	-			CTX-M-15							
20	*E. coli*	ESBL	Disc Diffusion	R	S	S	R(13)	R	R	R	R	R	R	-
			Hydrolysis	-			H	H	H	H	H	-	-	-
			Sequencing	TEM-198*			CTX-M-15							
21	*E. coli*	ESBL	Disc Diffusion	R	R	S	R (12)	R	R	R	R	R	R	-
			Hydrolysis				H	H	H	H	H	-	-	-
			Sequencing	TEM-198*			CTX-M-9 (gr-9)							
22	*E. coli*	ESBL	Disc Diffusion	R	R	S	R	R	R	R	R	R	R	-
			Hydrolysis	-			H	H	H	H	H	-	-	-
			Sequencing	OXA-1			CTX-M-15							

Strain No.	ID	Mechanism	Methods	Penicillins			Cephalosporin							
				AMO	AUG	TZP	CAZ	CTX	CPD	CRO	FEP	CL	CXM	FOX
23	*E. coli*	ESBL	Disc Diffusion	R	R	S	R	R	R	R	R	R	R	-
			Hydrolysis	-			H	H	H	H	H	-	-	-
			Sequencing	TEM-198*, OXA-1			CTX-M-15							
24	*E. coli*	ESBL	Disc Diffusion	R	S	S	R (20)	R	R	R	R	R	R	-
			Hydrolysis				H	H	H	H	H	-	-	-
			Sequencing	TEM-150*			CTX-M-15							
25	*E. coli*	ESBL	Disc Diffusion	R	S	S	R	R	R	R	R	R	R	-
			Hydrolysis	-			H	H	H	H	H	-	-	-
			Sequencing	-			CTX-M-15							
26	*E. coli*	ESBL	Disc Diffusion	R	S	S	R (14)	R	R	R	R	R	R	-
			Hydrolysis	-			H	H	H	H	H	-	-	-
			Sequencing	TEM-198*			CTX-M-9 (gr-9)							
27	*E. coli*	Ceph-Res but no ESBL/ AmpC	Disc Diffusion	R	S	S	R	R	R	R	R	R	R	-
			Hydrolysis				H	H	H	H	H	-	-	-
			Sequencing	TEM-198*			-							
28	*K. pneumoniae*	ESBL	Disc Diffusion	R	R	S	R (10)	R	R	R	R (15)	R	R	-
			Hydrolysis	-			H	H	H	H	H	-	-	-
			Sequencing	TEM-148*, OXA-1			CTX-M-15, SHV-27 (2be- extended spectrum)							
29	*E. coli*	ESBL	Disc Diffusion	R	S	S	R (18)	R	R	R	R (15)	R	R	-
			Hydrolysis	-			H	H	H	H	H	-	-	-
			Sequencing	TEM-105*			CTX-M-14							
30	*K. pneumoniae*	ESBL	Disc Diffusion	R	R	S	R (11)	R	R	R	R (15)	R	R	-
			Hydrolysis	-			H	H	H	H	H	-	-	-
			Sequencing	TEM-198*, OXA-1			CTX-M-15, SHV-27 (2be- extended spectrum)							

Strain No.	ID	Mechanism	Methods	Penicillins			Cephalosporin							
				AMO	AUG	TZP	CAZ	CTX	CPD	CRO	FEP	CL	CXM	FOX
31	*E. coli*	ESBL	Disc Diffusion	R	R	S	R	R	R	R	R	R	R	-
			Hydrolysis	-			SH	H	H	H	H	-	-	-
			Sequencing	OXA-1			CTX-M-15							
32	*E. coli*	Ceph-Res but no ESBL/ AmpC	Disc Diffusion	R	S	S	R (20)	R	R	R	R (14)	R	R	-
			Hydrolysis	-			H	H	H	H	H	-	-	-
			Sequencing	-			-							
33	*E. coli*	ESBL	Disc Diffusion	R	R	S	R	R	R	R	R (17)	R	R	
			Hydrolysis	-			H	H	H	H	H	-	-	-
			Sequencing	OXA-1			CTX-M-15							
34	*E. coli*	ESBL	Disc Diffusion	R	S	S	R (12)	R	R	R	R	R	R	
			Hydrolysis	-			H	H	H	H	H	-	-	-
			Sequencing	-			CTX-M-3							
35	*E. coli*	ESBL	Disc Diffusion	R	R	S	R	R	R	R	R (15)	R	R	-
			Hydrolysis	-			H	H	H	H	H	-	-	-
			Sequencing	OXA-1			CTX-M-15							
36	*E. coli*	ESBL	Disc Diffusion	R	S	S	R (18)	R	R	R	R (19)	R	R	-
			Hydrolysis	-			H	H	H	H	H	-	-	-
			Sequencing	TEM-198*			CTX-M-9							
37	*E. coli*	ESBL	Disc Diffusion	R	S	S	R (19)	R	R	R	R (18)	R	R	-
			Hydrolysis	-			H	H	H	H	H	-	-	-
			Sequencing	-			CTX-M-9							
38	*E. coli*	ESBL	Disc Diffusion	R	S	S	R (10)	R	R	R	R (18)	R	R	-
			Hydrolysis	-			H	H	H	H	H	-	-	-
			Sequencing	-			CTX-M-3							
39	*E. coli*	ESBL	Disc Diffusion	R	R	S	R	R	R	R	R (20)	R	R	-
			Hydrolysis	-			H	H	H	H	H	-	-	-
			Sequencing	OXA-1			CTX-M-15							

Strain No.	ID	Mechanism	Methods	Penicillins			Cephalosporin							
				AMO	AUG	TZP	CAZ	CTX	CPD	CRO	FEP	CL	CXM	FOX
40	*Enterobacter cloacae*	AmpC	Disc Diffusion	R	R	R	R	R	R	R	R (11)	R	R	-
			E-test (mg/L)	-			256	>256	-	>32	8	-	-	>256
			Hydrolysis	-			H	H	H	NH	NH	-	-	-
			Sequencing	-			ACT-14							
41	*E. coli*	ESBL	Disc Diffusion	R	R	S	R	R	R	R	R	R	R	-
			Hydrolysis	-			H	H	H	H	H	-	-	-
			Sequencing	OXA-1			CTX-M-15							
42	*E. coli*	ESBL	Disc Diffusion	R	S	S	R	R	R	R	R	R	R	-
			Hydrolysis				H	H	H	H	H	-	-	-
			Sequencing	TEM-76 (2br- inhibitor resistant)			CTX-M-15							
43	*Citrobacter braakii*	AmpC	Disc Diffusion	R	R	S	R	R	R	R	S (30)	R	R	R
			E-test (mg/L)	-			>256	64	-	>32	0.75	-	-	>256
			MALDI-TOF	-			H	H	H	NH	SH	-	-	
			Sequencing	-			-							
44	*E. coli*	Plasmid AmpC	Disc Diffusion	R	R	S	R	R	R	R	R (24)	R	R	R
			E-test (mg/L)	-			64	64	-	>32	0.75	-	-	>256
			MALDI-TOF	-			H	NRR	H	SH	H	-	-	
			Sequencing	TEM-122 (2br- inhibitor resistant)			CMY-42							
45	*E. coli*	Plasmid AmpC	Disc Diffusion	R	R	R	R	R	R	R	R (20)	R	R	R
			E-test (mg/L)	-			64	64	-	>32	2	-	-	>256
			MALDI-TOF	-			H	NH	H	NH	H	-	-	
			Sequencing	-			CMY-33							
46	*K. oxytoca*	AmpC	Disc Diffusion	R	R	S	R	R	R	R (12)	R (25)	R	R	R
			E-test (mg/L)	-			0.06	0.06	-	0.094	0.047	-	-	2
			Hydrolysis	-			H	H	H	H	H	-	-	
			Sequencing	OXY-2										

Strain No.	ID	Mechanism	Methods	Penicillins			Cephalosporin							
				AMO	AUG	TZP	CAZ	CTX	CPD	CRO	FEP	CL	CXM	FOX
47	*Enterobacter cloacae*	AmpC	Disc Diffusion	R	R	S	R	R	R	R	S (30)	R	R	R
			E-test (mg/L)	-			0.5	0.25	-	0.38	0.064	-	-	>256
			Hydrolysis	-			NH	NH	H	NH	H	-	-	
			Sequencing	-			-							
48	*Enterobacter aerogenes*	AmpC	Disc Diffusion	R	R	S	R (16)	R (17)	R	R (17)	S (30)	R	R	R
			E-test (mg/L)	-			0.5	0.12	-	0.125	0.094	-	-	>256
			Hydrolysis	-			NH	NH	SH	NH	NH	-	-	
			Sequencing	-			-							
49	*E. coli*	Plasmid AmpC	Disc Diffusion	R	R	R	R	R	R	R	R (18)	R	R	R
			E-test (mg/L)				>256	>256	-	>32	-	-	-	>256
			MALDI-TOF				NH	NH	SH	NH	H	-	-	
			Sequencing				CMY-44							
50	*Enterobacter aerogenes*	AmpC	Disc Diffusion	R	R	S	R	R	R	R	R (25)	R	R	R
			E-test (mg/L)	-			16	16	-	8	0.25	-	-	6
			Hydrolysis	-			NH	NRR	SH	SH	H	-	-	-
			Sequencing	-			-							
51	*E. coli*	Plasmid AmpC	Disc Diffusion	R	R	S	R (14	R (14)	R	R	R (26)	R	R	R
			E-test (mg/L)	-			32	32	-	16	1	-	-	>256
			Hydrolysis	-			NH	SH	SH	SH	H	-	-	-
			Sequencing	TEM-198			CMY-2							

Phenotypic method based on the BSAC guideline. Sequencing profiles (ESBL and *ampC* genes) were found with identity >90%. Other AmpC variants probably of chromosomal origin were detected at cut off < 90%.

Legend: R- resistant, S- susceptible**,** H- hydrolysis, NH- no hydrolysis, NRR - no reliable results, - disagreement between phenotypic results and hydrolysis assays, - disagreement between disc diffusion test and E-test, SH-slow hydrolysis

3.3 Multiplex tandem PCR (MT-PCR) for detection of bacterial resistance genes in infected urines and isolates

AIMS:

- To develop and evaluate a test to detect the commonest antibiotic resistance genes among uropathogenic Enterobacteriaceae.

The intention was to develop an assay that could be used, together with MALDI-TOF for pathogen identification to give a comprehensive and rapid evaluation of the pathogen's resistances for high-risk urosepsis patients.

Three successive iterations of the MT-PCR test were designed: (i) the Easy-Plex 8-Plex assay to detect 8 antibiotic resistance genes including the four commonest trimethoprim resistance determinants (*dfrA1, A5/A14, A7/A17, A12)*, two aminoglycoside genes (*aadA1/A2/A3* and *aac(6')-Ib*) conferring streptomycin and tobramycin resistance respectively, and two fluoroquinolone related genes (*gyrA* and *KPparC*) (see Table 7 Section 2.3.1); (ii) the High-Plex 16-Plex assay to identify 16 resistance genes including the eight targets represented previously and additional targets for β-lactamase genes, specifically: ESBLs ($bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-9}}$), AmpC (bla_{CMY}), penicillinases (bla_{TEM}, bla_{SHV}, $bla_{\text{OXA-1}}$), carbapenemases ($bla_{\text{OXA-48}}$, bla_{KPC}, bla_{NDM}, bla_{VIM}), and (iii) the Easy-Plex 24-Plex assay to detect 24 target genes including the 16 targets from the previous assay and additional targets to seek four further aminoglycoside genes (*aadB, aacC1, aacC2, aacC3*) responsible for gentamicin and/or tobramycin resistance; four additional targets were also included to identify non Enterobacteriaceae to genus level.

3.3.1 Easy-Plex (8-Plex) assay for antibiotic resistance gene detection

A total of 21 samples in three PCR assays were tested using reference strains from PHE and three clinical urines in four different formats: (i) extracted DNA (n=7), (ii) pure bacterial colonies taken from culture plates (n=7), (iii) urine spiked with known organism (n=5), and (iv) clinical urines (n=2) (see section 2.3.1). The results were compared with phenotypic susceptibility testing based on the BSAC methodology and sequencing, which was performed for the reference strains only (see Table 34).

Trimethoprim genes (*dfrA1 or A5/A14 or A7/A17 or A12)* were found in all 21 irrespective of format (DNA, cultivated isolates, urine spiked or clinical urines) tested samples, congruent with an observed phenotypic trimethoprim resistance. Sequencing data (available for 18 samples) confirmed the presence of the *dfr* genes found by the Easy-Plex assay.

The aminoglycoside gene *aadA* (*aadA1/A2/A3*) encoding an adenyltransferase was found in 8 out of the 21 analysed samples. These include 5 out of 14 streptomycin-resistant isolates, and 3 out of 7 streptomycin-susceptible isolates. Sequencing confirmed 7 out these 8 *aadA1/A2/A3* PCR positive variants. Disagreements here may be explained by the frequent presence of other genes or mutations conferring streptomycin resistance, and by a strain having but not expressing *aadA.*

The assay found *aac(6')-Ib*, encoding an aminoglycoside 6'-N-acetyltransferase, in 14 out of 21 examined samples, agreed with tobramycin resistance and sequencing (for the 18 cases where data were available).

Either fluoroquinolone *gyr A1/S* or *A1/R* products were detected in all *E. coli* (n=14) whilst *gyr KlebR/S* and *KPparC* products were obtained for all *K. pneumoniae* (n=7). Variation in melting temperature was seen for the *E. coli gyr/A1* product: this was in a range 85-86^{o}C (mean 85.5 ^{o}C) for *E. coli* with ciprofloxacin-resistance but 86-87^{o}C (mean 86.5 ^{o}C) for ciprofloxacin-susceptible *E. coli*; for *Klebsiella* the T_m was in a range 88-89^{o}C (mean 88.5 ^{o}C) irrespective of ciprofloxacin resistance or susceptibility. The test thus allowed discrimination between ciprofloxacin-susceptible and -resistant *E. coli*, but not for *Klebsiella* spp.

Table 34. Comparison between Easy-Plex (8-Plex) assay and reference methods.

Reference methods (phenotypic testing and/or sequencing)	**PCR assay +ve results**				**PCR assay -ve results**			
	Trimethoprim (n=21)							
	dfrA1	*dfrA5/ A14*	*dfrA7/ 17*	*dfrA12*	*dfrA1*	*dfrA5/A 14*	*dfrA7/ 17*	*dfrA12*
Gene sequenced & Trim R	3	5	7	3	0	0	0	0
Trim R*	1	1	1	0	0	0	0	0
Trim S	0	0	0	0	0	0	0	0
	Aminoglycoside (n=21)							
	aadA1/A2/A3				*aadA1/A2/A3*			
Gene sequenced & Strep R	4				0			
Strep R*	1				9			
Gene sequenced & Strep S	3				0			
Strep S*	0				4			
	aac(6')-Ib				*aac(6')-Ib*			
Gene sequenced & Tobra R	13				0			
Tobra R*	1				0			
Gene sequenced & Tobra S	0				5			
Tobra S*	0				2			
	Fluoroquinolone (n=21)							
	E. coli gyrA1/R		*E. coli gyrA1/S*		*K. pneumoniae Kleb R/S*		*K. pneumoniae KPparC*	
Cipro R	10		0		6		6	
Cipro S	0		4		1		1	

Legend: R- resistant, S- sensitive, * phenotypic data available only, not sequence.

3.3.2 High-Plex (16-Plex) assay for antibiotic resistance genes detection

The assay was performed directly on 74 clinical urines from the NNUH and 35 cultivated isolates, without DNA extraction. The isolates were from PHE and NNUH. Performance was similar regardless of whether urines or cultured bacteria were used.

Resistance genes were confirmed on the basis of (i) a high number of molecules (> 1000 copies) in the 2nd PCR step, (ii) a cycle threshold (C_T) < 20 in the 2nd PCR step, (iii) a correct melting temperature for the amplified gene (T_m). Output reads for a representative urine, tested directly without culture, are shown in Figure 60. The High-Plex results were compared with phenotypic susceptibility data, sequencing and real-time PCR on isolated bacteria, according to the particular resistance, as presented in Tables 35 and 36. Sensitivity and specificity was calculated using the online calculator and results are presented in Table C in the Appendix C. Although positive and negative predictive values were also calculated we concentrated on sensitivity and specificity as fundamentally identifying how well a test identifies samples with positive and negative results rather than assessing probabilities, which are likely to be affective by the original sample selection.

3.3.2.1 Tests directly on urines

Identification of β-lactamase genes directly from clinical urines was in a good agreement with real-time PCR. Thus, when used directly on urines, the assay detected following extended-spectrum β-lactamases (24 *bla*$_{\text{CTX-M gp 1,}}$ 13 *bla*$_{\text{CTX-M gp 9}}$), AmpC (9 *bla*$_{\text{CMY}}$), and penicillinases (33 *bla*$_{\text{TEM,}}$ 6 *bla*$_{\text{SHV,}}$ 8 *bla*$_{\text{OXA-1}}$) with 95.3% - 100% specificity and 100% sensitivity.

Trimethoprim determinants (*dfrA1/A5/A7/A12*) were found in 39 out of the 41 urines containing isolates resistant to trimethoprim, with sensitivity calculated as 92.7% and specificity 97%.

Among aminoglycoside determinants *aac(6')-Ib* encoding 6'-N-acetyltransferase, was identified in 9 urines compared with 8 urines containing bacteria resistant to tobramycin with 100% sensitivity and 98.5% specificity. It is inferred that the gene

probably was not expressed in one case. *aadA* (*aadA1, aadA2, aadA3*), encoding streptomycin adenyltransferases was detected in 9 out of 24 urines containing bacteria resistant to streptomycin, but also in 8 out of 50 urines containing bacteria susceptible to streptomycin with the lowest sensitivity 37.5%, and specificity 86%. This lack of concordance, similar as seen in the 8-Plex assay, implies (i) the frequent presence of other unsought genes or mutations causing streptomycin resistance (e.g. *aadA5* or *strA/strB*), and (ii) variable expression of *aadA* gene cassettes within integrons, where this gene commonly exists and its expression may vary with distance from the promotor.

3.3.2.2 Cultivated isolates

Detection of β-lactamase genes by the Easy-Plex in cultivated isolates perfectly agreed with reference molecular methods giving 100% sensitivity and specificity. Enzymes found included: ESBLs (18 $bla_{\text{CTX-M gp 1,}}$ 4 $bla_{\text{CTX-M gp 9}}$), AmpC (7 bla_{CMY}), penicillinases (24 $bla_{\text{TEM,}}$ 16 $bla_{\text{SHV,}}$ 18 $bla_{\text{OXA-1}}$) and carbapenemases (7 $bla_{\text{OXA-48}}$, 5 bla_{KPC}, 10 bla_{NDM}, 1 bla_{VIM}) genes, all in perfect agreement to reference real-time PCR and sequencing.

Trimethoprim determinants (*dfrA1/A5/A7/A12*) were identified in 30 out of 32 trimethoprim-resistant isolates with near identical sensitivity and specificity (93.7%, 100%) to that found when urines were tested directly. It Is likely that the two isolates with negative results had other rare *dfr* variants.

aac(6')-Ib was found in 22 out of the 24 isolates with phenotypic tobramycin resistance. The sensitivity was lower compared with urines (100% *vs.* 91.7%) and specificity was 100%. *aadA* determinants were detected in 10 out of 18 streptomycin-resistant isolates and in 6 out of 17 streptomycin-susceptible isolates; once again, these mis-matches were similar to those seen for urines, with sensitivity 55.7% and specificity 64.7%.

The High-Plex UTI assay was designed primarily to detect antibiotic resistance genes; however review of the melting temperature for the 'Enterobacteriaceae' gene together with the melting temperature for *gyr* product potentially enabled identification to genus level (see Figure 66). Further identification to species level was

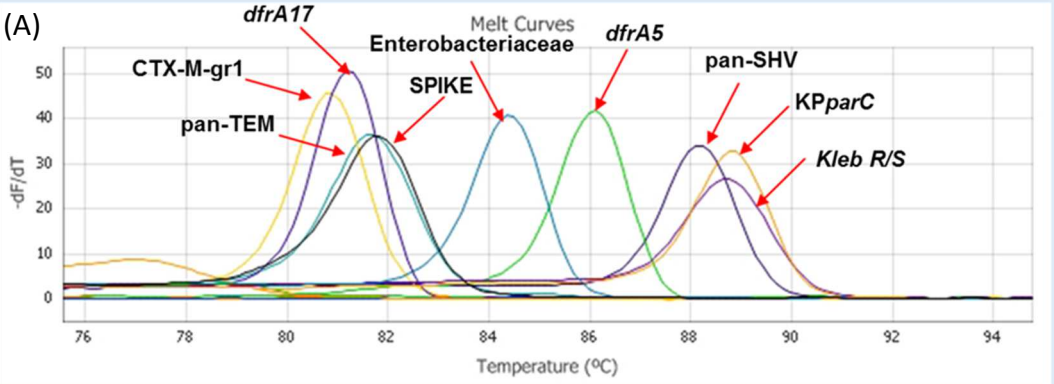
performed on MALDI-TOF using pure colony growth on agar plates. Among 109 examined pathogens there were 66 *E. coli*, 20 *K. pneumoniae* and 19 other Enterobacteriaceae species including: *C. freundii* (n=3), *E. aerogenes* (n=10), *S. marcenscens* (n=3), *K. oxytoca* (n=2) and *Proteus* spp. (n=1), also one *Acinetobacter* spp., and three *Pseudomonas* spp.

The fluoroquinolone *gyr A1/R* gene was identified in 25 out of 28 *E. coli*-containing urines, and in 9 out of the 10 *E. coli* cultured clinical isolates with ciprofloxacin-resistance, whereas *gyr A1/S* was found in 22 urines containing ciprofloxacin-susceptible *E. coli* and in 6 *E. coli* isolates susceptible to ciprofloxacin, and also in 3 urines and 1 isolate with ciprofloxacin-resistant *E. coli*. The *gyr Kleb R/S* product was detected in all 15 *K. pneumoniae* isolates and all 5 *K. pneumonia*-containing urines; resistant and susceptible profiles were not discriminated. The three *gyr* types: *gyr A1/R, A1/S and Kleb R/S* were variously identified in 19 urines and isolates belonging to other Enterobacteriaceae species, and in 4 urines and isolates containing non-fermenters, always with little or no discrimination between ciprofloxacin susceptible or resistant organisms.

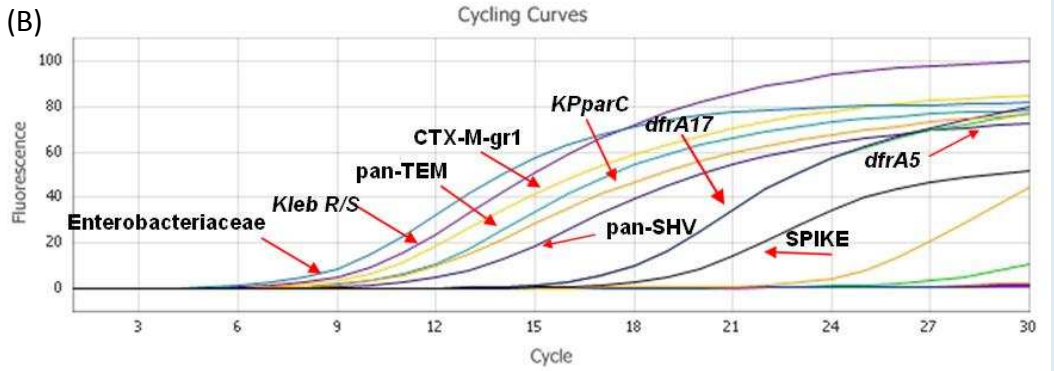
The *KPparC* gene was found in 21 urines and 17 isolates comprising 2/3 *Citrobacter* spp., 9/10 *Enterobacter* spp., 2/2 *K. oxytoca,* 20/20 *K. pneumoniae*, 3/3 *Serratia* spp., and in the single *Proteus* spp. and *Acinetobacter* whereas no signal was seen for any of the 66 *E. coli* and 3 *Pseudomonas* spp. There was no differentiate in T_m between amplification products susceptible and resistant isolates.

In summary, fluoroquinolone -susceptible and -resistant *E. coli,* but not other Gram-negative bacteria were distinguished by the melting temperatures of the amplification products from the *gyr* genes (*gyrA1/R* or *gyrA1/S*) (Table 36, Figure 61) using both urines and cultivated isolates. Time from specimen to results was *c.* 3.5 hours.

(A)



(B)



(C)

Colour	Gene	Call	Number [copies of molecules]	T_m [°C]	C_T
	pan-TEM	+	2,032,360	81.67	7.83
	pan-SHV	+	1,190,321	88.18	8.66
	CTX-M gr 1	+	4,175,992	80.84	6.71
	CTX-M gr 9				
	pan-CMY				
	OXA-1				
	OXA-48				
	dfrA	+ (dfrA5)	36,268	86.09	14.1
	dfrA	+ (dfrA17)	35,502	81.22	14.14
	aminoglycosides				
	KPC				
	VIM/NDM				
	gyrA1	+ (Kleb R/S)	5,854,436	88.71	6.18
	KPparC	+	2,907,402	88.82	7.27
	Enterobacteriaceae	+	10,423,897	84.39	5.28
	SPIKE	+	10,000	81.8	16.11

Figure 60. High-Plex UTI assay using clinical urine containing *K. pneumoniae*. (A) Melting temperature curves, (B) Cycle curves, (C) Antibiotic.

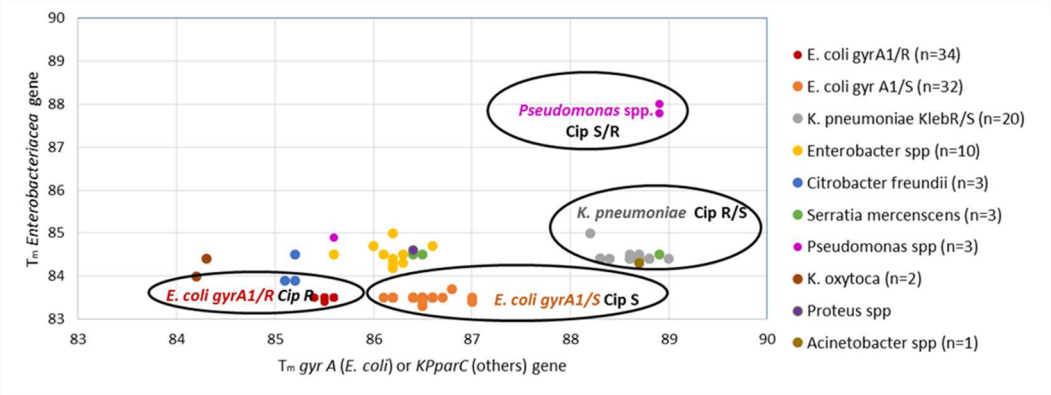


Figure 61. Melting ranges for *gyr* and 'Enterobacteriaceae' genes in the High-Plex UTI assay as an aid to genus identification.

Table 35. Sensitivity and specificity of High-Plex UTI assay.

Resistance Gene Target	**Clinical urines (n=74)**			**Isolates (n=35)**		
β-Lactamase genes vs. molecular reference genes detection by real-time PCR						
	High-Plex assay	Reference method	Sensitivity & Specificity	High-Plex assay	Reference method	Sensitivity & Specificity
bla$_{TEM}$	33	31	100%; 95.3%	24	24	100%; 100%
bla$_{SHV}$	6	6	100%; 100%	16	16	100%; 100%
bla$_{CTX-M\ gr1}$	24	23	100%; 98%	18	18	100%; 100%
bla$_{CTX-M\ gr9}$	13	13	100%; 100%	4	4	100%; 100%
bla$_{CMY}$	9	9	100%; 100%	7	7	100%; 100%
bla$_{OXA-1}$	8	8	100%; 100%	18	18	100%; 100%
bla$_{OXA-48/181/244}$	-	-	-	7	7	100%; 100%
bla$_{KPC}$	-	-	-	5	5	100%; 100%
bla$_{NDM}$	-	-	-	10	10	100%; 100%
bla$_{VIM}$	-	-	-	1	1	100%; 100%
Trimethoprim gene vs. phenotypic trimethoprim resistance						
dfrA1/A5/A7/A12	39	41	92.7%; 97%	30	32	93.7%; 100%
Aminoglycoside genes vs. phenotypic tobramycin and streptomycin resistance						
aac(6')-Ib	9	8	100%; 98.5%	22	24	91.7%; 100%
aadA1/A2/A3	9	24	37.5%; 86%	10	18	55.7%; 64.7%

Table 36. Detection of gyrase identification by the High-Plex UTI assay in relation to bacterial species.

	Fluoroquinolone genes												
Pathogens (n=109)	*gyrA1/R*				*gyrA1/S*				*gyr KlebR/S*				*KPparC*
	Cipro R		Cipro S		Cipro R		Cipro S		Cipro R		Cipro S		
	U	I	U	I	U	I	U	I	U	I	U	I	
E. coli (n=66)	25	9	-	-	3	1	22	6	-	-	-	-	-
K. pneumoniae (n=20)	-	-	-	-	-	-	-	-	4	13	1	2	20
Non-Enterobacteriaceae (n=4)*			1							1	2		2
Other Enterobacteriaceae species (n=19)**	1		4	1	1		8	3			1		16

Legend: R- resistant, S- susceprible, U-urine, I- isolate.***** *Acinetobacter* spp. (n=1), *Pseudomonas* spp. (n=3); ** *Citrobacter freundii* (n=3), *Enterobacter aerogenes* (n=10), *Serratia marcenscens* (n=3), *Klebsiella oxytoca* (n=2), *Proteus* spp. (n=1).

3.3.3 Easy-Plex (24-Plex) assay for antibiotic resistance gene detection and bacterial genus identification

The 24-Plex assay was designed to seek further common aminoglycoside resistance genes in Enterobacteriaceae, responsible for tobramycin or/and gentamicin resistance. The expanded panel included the aminoglycoside determinants *aac(3)-I* (*aacC1*) encoding AAC(3)-I enzyme for gentamicin resistance, *aac(3)-II* (a and d variants), *aac(3)-III*, and *ant(2")-I* (*aadB*) encoding AAC(3)-II, AAC(3)-III, and ANT(2")-I enzymes conferring resistance to both gentamicin and tobramycin.

A total of 23 samples were run. These include 16 infected clinical urines and 7 bacterial isolates. Resistance genes were confirmed on the basis of (i) a high number of molecules (>550 copies of molecules) in the 2nd PCR step, (ii) a cycle threshold (C_T) ≤ 21 in the 2nd PCR step, (iii) a correct melting temperature for the gene product (T_m). Results were variously compared with reference methods: (i) sequencing using the Illumina platform, performed for isolates only, (ii) real time PCR with SyberGreen to seek ESBL genes and (iii) disc diffusion susceptibility testing by BSAC methodology. A comparison between the 24-Plex assay and reference methods is presented in Table 37.

Among 23 tested specimens, Easy-Plex detected 12 *E. coli* and 11 *K. pneumoniae* agreeing with culture and MALDI-TOF results both for directly tested urines and cultured isolates. Specifically, the melting temperature for the 'Enterobacteriaceae' target, together with the melting temperature for the *gyr* product enabled differentiation of *E. coli* and *K. pneumoniae.* In 14 out of 16 directly-tested urines the Easy-Plex additionally detected 6 enterococci and streptococci.

3.3.3.1 Directly tested urines

Among the 16 infected clinical urines the assay found genes for extended-spectrum β-lactamases (11 $bla_{\text{CTX-M gp 1,}}$ 3 $bla_{\text{CTX-M gp 9}}$) and penicillinases (11 $bla_{\text{TEM,}}$ 6 $bla_{\text{SHV,}}$ 8 $bla_{\text{OXA-1}}$). In general, there was a good agreement to molecular reference methods, but two cases of each $bla_{\text{TEM,}}$ $bla_{\text{CTX-M gp-1}}$ and $bla_{\text{OXA-1}}$ were additionally found among the urines, not confirmed by real-time PCR.

Trimethoprim determinants (*dfrA1/A5/A7/A12*) were detected in 10 out of 13 urines containing bacteria resistant to trimethoprim. Negative results were obtained for 3 urines with trimethoprim resistant bacteria; 2 out of these 3 failures nonetheless gave weak signals for *dfrA5/A14* (concentration < 230; C_T value >22, signal present but poor amplification); the remaining urine was negative by the PCR assay for all acquired *dfr* genes sought, suggesting the presence of some rarer or novel trimethoprim resistance determinant.

Overall, 12/14 urines contained bacteria resistant to tobramycin and all these bacteria were also gentamicin-resistant. Three out of these 14 urines contained bacteria that had more than one aminoglycoside resistance gene whilst 5 had only one aminoglycoside gene and 6 were negative by the Easy-Plex, although weak signals were seen for *aac(6')-Ib* (n=4)*,* a*ac3'-II* (*aacC2*) (n=5), and a*ac3'-I* (*aacC1*) genes (n=1) in several of these. All the positive results found by Easy-Plex agreed with phenotypic susceptibility testing.

In term of specific genes, *aac(6')-Ib* was found in 5 out of the 12 urines containing bacteria resistant to tobramycin*;* 4 out of the 7 negatives also gave a weak signal (concentration in a range 100-435; C_T value >22). *aadB* was detected in one urine and a*ac3'-II* (*aacC2*) in 5 urines containing bacteria resistant to both gentamicin and tobramycin. *aac(3')-I* (*aacC1*) and *aac(3')-III* (*aacC3*) genes were not found by Easy-Plex in any of the urines, except that a weak signal (concentration 86; C_T value >24) was seen for *aac3'-I* in one urine containing bacteria resistant to gentamicin and tobramycin.

The final aminoglycoside determinant *aadA,* conferring streptomycin resistance, was found in one out of 7 urines containing bacteria resistant to streptomycin. This lack of concordance, noted already for earlier iterations of the test, indicates the presence of other genes or mutations responsible for streptomycin resistance but not sought in this panel (particularly *strA/strB* or mutations).

3.3.3.2 Testing of cultured isolates

The identification of β-lactamase genes in cultured clinical isolates mostly agreed with reference molecular methods, except for *bla*$_{TEM}$ where MT-PCR assay detected 2 additional cases. Thus, the following β-lactamase genes were found: ESBLs (6 *bla*$_{CTX\text{-}M\ gp\ 1}$, 1 *bla*$_{CTX\text{-}M\ gp\ 9}$), *ampC* (3 *bla*$_{CMY}$), penicillinases (9 *bla*$_{TEM}$, 4 *bla*$_{SHV}$, 6 *bla*$_{OXA\text{-}1}$), and carbapenemases (1 *bla*$_{OXA\text{-}48}$, 1 *bla*$_{KPC}$, 2 *bla*$_{NDM}$, 1 *bla*$_{VIM}$).

Trimethoprim (*dfrA1/A5/A7/A12*) genes were found in 6 out of 8 isolates with phenotypic trimethoprim resistance; 2 negatives gave weak signal for *dfrA5/A14* (concentration < 230; C_T value >22) and sequencing confirmed the presence of *dfrA14* gene in both these 2 isolates. Sequencing also found two trimethoprim determinants in two other isolates (*dfrA1* and *dfrA14*; *dfrA17* and *dfrA12*) but Easy-Plex detected only one *dfr* gene in each sample (*dfrA14* and *dfrA12*, respectively).

In total, 8 out of 9 isolates were tobramycin and gentamicin resistant. Seven out of these 8 resistant isolates had more than one aminoglycoside resistance gene, and the remaining one gave a weak signal for a*ac3'-II* (*aacC2*). *aac(6')-Ib* was found in 6 out of 8 tobramycin-resistant isolates, congruent also with sequencing; *aadB* was identified in 2 isolates associated with both gentamicin and tobramycin resistance, also agreeing with sequencing data; a*ac3'-II* (*aacC2*) was detected in 6/8 isolates with phenotypic tobramycin and gentamicin resistance, again perfectly agreed with sequencine data. Two other isolates that were gentamicin and tobramycin resistant gave weak signals (concentration 61 and 72; C_T value >24), also confirmed by sequencing. *aac(3')-I* (*aacC1*) and *aac(3')-III* (*aacC3*) genes were never found by the Easy-Plex assay, although sequencing detected *aac3'-I* type b and *aac3'-I* type e in two isolates: one with phenotypic resistance to gentamicin and tobramycin, the other susceptible to these antibiotics. The later result suggests that *aac3'-I* was not expressed in this case. Similar as for urine *aadA* was detected in one out of 5 streptomycin-resistant isolates implying the presence of other genes or mutations associated with streptomycin resistance in the remaining four.

The *gyr A1/R* gene was identified in 7/14 urines containing ciprofloxacin resistant *E. coli* and in 3/9 resistant *E. coli* isolates whereas *gyr A1/S* was found in one *E. coli* -containing urine and one *E. coli* isolate with phenotypic ciprofloxacin resistance. The

gyr Kleb R/S was detected in 6 urines containing 5 ciprofloxacin-resistant and one ciprofloxacin-susceptible *K. pneumoniae* and in 5 isolates containing 4 ciprofloxacin-resistant and one ciprofloxacin-susceptible *K. pneumoniae*. The *KPparC* gene was found in 10/11 *K. pneumoniae* specimens (6 urines and 4 isolates) and never in *E. coli.*

Overall, the 24-Plex assay was less discriminatory than the 16-Plex assay in detecting antibiotic resistance genes. Several of the gene targets e.g *aac(6')-Ib,* a*ac3'-II* or *dfrA5/A14* often showed weak signals for several isolates or infected urines, below the cut off value applied, but with the presence of the genes confirmed by sequencing. Timeframe from specimen to results was shorten than 16-Plex assay at *c.* 2 h.

Table 37. Comparison between 24-Plex assay and reference method.

(A) Antibiotic resistance gene detection

Resistance Gene Target	**Clinical urines (n=14)**		**Isolates (n=9)**	
β-Lactamase genes vs. reference (gene detection by PCR or sequencing)				
	High-Plex assay	Reference method	High-Plex assay	Reference method
bla$_{TEM}$	11	9	9	7
bla$_{SHV}$	6	6	4	4
bla$_{CTX-M\ gr1}$	11	9	6	6
bla$_{CTX-M\ gr9}$	3	3	1	1
bla$_{CMY}$	0	0	3	3
bla$_{OXA-1}$	8	6	6	6
bla$_{OXA-48/181/244}$	0	0	1	1
bla$_{KPC}$	0	0	1	1
bla$_{NDM}$	0	0	2	2
bla$_{VIM}$	0	0	1	1
Trimethoprim gene vs. phenotypic trimethoprim resistance			Trimethoprim gene vs. phenotypic trimethoprim resistance and sequencing	
dfr genes (pooled samples)	10	13	6	8
including:				
dfrA1,	3		0	1
dfrA5/A14,	4		2	4
dfrA7/A17,	5		3	4
dfrA12	0		1	1
Aminoglycoside gene vs. streptomycin resistance			Aminoglycoside gene vs. streptomycin resistance and sequencing	
aadA1/A2/A3	1	7	1	5
Aminoglycoside gene vs. tobramycin resistance			Aminoglycoside gene vs. tobramycin resistance and sequencing	
aac(6')-Ib	5	12	6	8
Aminoglycoside genes vs. tobramycin and gentamicin resistance			Aminoglycoside genes vs. tobramycin and gentamicin resistance and sequencing	
aadB, aac3'-I, aac3'-II, aac3'-III (pooled samples)	5	14	7	8
including:				
aadB	1		2	2
aac3'-I	0		0	2
aac3'-II	5		6	8
aac3'-III	0		0	0

(B) Pathogens and gyrase I/D

	Fluoroquinolone genes						
Pathogens (n=23)	*gyrA1/R*		*gyrA1/S*		*gyr KlebR/S*		*KPparC*
E. coli (n=12)	Cipro R	Cipro S	Cipro R	Cipro S	Cipro R	Cipro S	0
	10	0	2	0	0	0	
K. pneumoniae (n=11)	0	0	0	0	9	2	10

3.3.3.3 Translating gene results to treatment guidelines

An attempt was made to translate Easy-Plex results to antibiotic choices using the algorithm shown in Table 9 in Section 2.3.3.

Figure 62 (A) and (B) present the output reads with suggested 'diagnosis'.

Sample one contains *E. coli* producing ESBL with $bla_{\text{CTX-M gr1}}$ detected. bla_{TEM} was also found but would not further expand resistance in the presence of a CTX-M gr1 enzyme. The pathogen was resistant to ciprofloxacin as inferred by the presence of *gyrA1/R*. Because *dfr* sought genes were not detected congruent with trimethoprim-susceptible profile, the software suggested to use of trimethoprim.

Specimen two again contain *E. coli*. Again, the Easy-Plex detected the extended-spectrum β-lactamase gene $bla_{\text{CTX-M gr1}}$ and bla_{TEM} penicillinase gene. In contrast to the first sample, PCR detected *dfrA1* indicating resistance to trimethoprim with agreement to phenotypic profile and *gyrA1/S,* indicating susceptibility to ciprofloxacin, also congruent with phenotypic profile. The assay also found *aadA1*, implying streptomycin resistance, however this agent is rarely used in the treatment of UTIs. None of the other aminoglycoside-modified enzyme genes were found indicating likely susceptibility to tobramycin, gentamicin and amikacin also confirmed by phenotypic method; therefore the software suggested that the patient may respond to gentamicin.

In both cases the Easy-Plex detected the presence of additional targets e.g. enterococcus or streptococcus or *dfrA17* gene (example B), but the number of molecules ('concentration') was below the threshold value (>550 copies of

molecules). As above (Section 3.3.3) we believe that some of these are likely to be true positive.

The software was desinged to interpret the results from the 24-Plex assay. However, in several cases we observed that it suggested an antibiotic e.g. gentamicin even though a gene conferring resistance to this antibiotic was found. Therefore the software needs to be improved to better guide and indicate treatment choices. Nonetheless, it is a first step to translating molecular results into clinical information to guide treatment.

(A)

No.	Sample	Gene	Call	Concentration
A1	64440	Enterobacteriac...	Present	1,522,704
B1	64440	Enterococcus	Present	29
C1	64440	Strep		
D1	64440	Staphylococcus		
E1	64440	pan-Pseudomonas	Present	5
F1	64440	oxa 1		
G1	64440	OXA-48 like		
H1	64440	dfrA		
A2	64440	dfrA_		
B2	64440	aminoglycosides		
C2	64440	KPC		
D2	64440	vim/NDM		
E2	64440	gyrA1	Present (gyrA1-R)	119,800
F2	64440	KP parC		
G2	64440	pan-TEM	Present	485,270
H2	64440	pan-SHV		
A3	64440	CTX-M group 1	Present	398,082
B3	64440	CTX-M group 9		
C3	64440	pan-CMY		
D3	64440	aadB		
E3	64440	aacC1		
F3	64440	aacC3		
G3	64440	aacC2		

Diagnosis

Patient may respond to oral trimethoprim

(B)

No.	Sample	Gene	Call	Concentration
A4	64157	Enterobacteriac...	Present	80,661
B4	64157	Enterococcus	Present	27
C4	64157	Strep	Present	84
D4	64157	Staphylococcus	Present	19
E4	64157	pan-Pseudomonas	Present	6
F4	64157	oxa 1		
G4	64157	OXA-48 like		
H4	64157	dfrA	Present (dfrA1)	140,020
A5	64157	dfrA_	Present (dfrA17)	103
B5	64157	aminoglycosides	Present (aadA1)	25,098
C5	64157	KPC		
D5	64157	vim/NDM		
E5	64157	gyrA1	Present (gyrA1-S)	11,692
F5	64157	KP parC		
G5	64157	pan-TEM	Present	24,237
H5	64157	pan-SHV		
A6	64157	CTX-M group 1	Present	13,458
B6	64157	CTX-M group 9		
C6	64157	pan-CMY		
D6	64157	aadB		
E6	64157	aacC1		
F6	64157	aacC3		
G6	64157	aacC2		

Diagnosis

Patient may respond to i.v. Gentamycin

Figure 62. Output reads for 24-Plex assay provided by prototype software.

3.4 MinION Nanopore sequencing

AIMS

- To evaluate MinION nanopore sequencing for pathogen identification and antibiotic resistance gene detection directly from clinical urines.

3.4.1 MinION results and performance improvement

Fifteen MinION runs were performed: 10 for culture-positive clinical urines, four using urine spiked with multi-resistant *E. coli* H141480453 and one for urine spiked with *E. coli* recovered from Clinical Urine 6 (Table 38). The first four runs, with Clinical Urines 1-4 and R7.0 chemistry flow cells, gave unsatisfactory results, failing even to deliver pathogen identification. These failures reflected inadequate human DNA depletion (CU1), poor quality of flow cells (CU2 and CU4) and low yields of bacterial DNA, caused by DNA degradation as a result of freezing. Thereafter, further development in the sample and library preparation procedure, together with new flow cells using R7.3 chemistry, led to progressive improvement in the quality and quantity of the sequence yields. This is illustrated in Figure 63, showing analysis based on 6 hours of sequencing. Data on the quality and quantity of bacterial DNA extracted from Clinical Urines 4-10, and all spiked urines, are presented in Figure D1 (Appendix D).

From Clinical Urine 5 (CU5) onwards, MinION sequencing produced 22968-141511 reads per run (counted as the total number of 1-D template reads, 1-D complement reads, and 2-D template + complement reads) with a mean read-length, varying with the run, in the range 2827-5419 bp. Between 6536-34330 reads compromised 2-D sequencing reads, of which 2518-22405 were classified as a "Pass 2-D reads," with a mean read-length of 3452 to 6076 bp, and a maximum length of 46213 bp (urine spiked with *E. coli* from Clinical Urine 6). The total number of reads classified as “Fail 2-D reads” per run was in the range 3749-9776, with a mean read-length of 2467-5421 bp. The manufacture claimed that single-read identity to

reference sequences improved from 70% to 85% during the course of these studies. Runs with R7.3 chemistry were processed for 7.5 to 48h (Table 38).

Successive runs with urine spiked with *E. coli* H141480453 illustrate significant improvement in coverage depth during the first 6 hours of sequencing, as presented in Figure 64. The first improvement, between Run 1 in 10/2014 and Run 2 in 05/2015 was in yield and depth of coverage. The second improvement, from Run 2 to Run 3, was achieved using WIMP and ARMA software for real-time analysis of sequence data, reducing the total processing time from specimen submission and library preparation to resistance gene profile in 7.5 h. The final improvement, from Run 3 to Run 4, was achieved using the Rapid Library Preparation Kit, identifying resistance gene in a timeframe similar to PCR (< 4 hours); however this run generated only 1-D reads that could not be analysed with automated WIMP/ARMA software, and had to be processed manually against the BLAST/CARD databases, as illustrated in Figure 65.

Table 38. Clinical and spiked urines subjected to MinION sequencing in chronological order.

Sample and date	Flow cell chemistry	Sequencing time (h)	Total number of reads	Mean readlength (bp)	Number of 2-D reads	Number of 2-D 'pass' reads	Mean readlength of 2-D 'pass' (bp)	Total number of 2-D 'fail' reads	Mean readlength of 2-D 'fail' (bp)
CU1 09-07-2014	R7.0	24	12295	3647	1645	0	0	0	0
CU2 12-07-2014	R7.0	24	8299	2859	621	0	0	0	0
CU3 04-09-2014	R7.0	No results							
CU4 09-09-2014	R7.0	21	3829	1728	184	0	0	0	0
Urine spiked with *E. coli* H141480453 run 1; 06-11-2014	R7.3	30	45652	2827	15216	10109 (66%)	4103	5107	3880
CU5 16-01-2015	R7.3	25.5	22968	3292	8191	2518 (26.5%)	3980	5673	3491
CU6 24-01-2015	R7.3	23	57289	4700	15932	12183 (48%)	5510	3749	4848
CU7 05-02-2015	R7.3	17.5	76499	4473	17050	10137 (18.8%)	5414	9776	4447
CU8 02-03-2015	R7.3	33	86294	4664	20799	13798 (36%)	5324	7001	4221

Sample and date	Flow cell chemistry	Sequencing time (h)	Total number of reads	Mean readlength (bp)	Number of 2-D reads	Number of 2-D 'pass' reads	Mean readlength of 2-D 'pass' (bp)	Total number of 2-D 'fail' reads	Mean readlength of 2-D 'fail' (bp)
Urine spiked with *E. coli* from CU6 09-03-2015	R7.3	14	56394	5419	13206	7678 (27.9%)	6076	5528	5421
CU9 30-03-2015	R7.3	26	28 767	4 926	6536	4376 (29%)	5741	2160	4572
Urine spiked with *E. coli* H141480453 run 2; 04-05-2015	R7.3	48	138 720	4 424	33589	17123 (27.7%)	5013	16466	4040
CU10 16-05-2015	R7.3	35	141 511	3 107	34330	15074 (23%)	3452	19256	2908
Urine spiked with *E. coli* H141480453 run 3; 23-10-2015	R7.3	7.5	97961	4308	28787	22405 (77%)	4416	6382	2467
Urine spiked with *E. coli* H141480453 run 4; 26-01-2016	R7.3	29	21441	2043	-	-	-	-	-

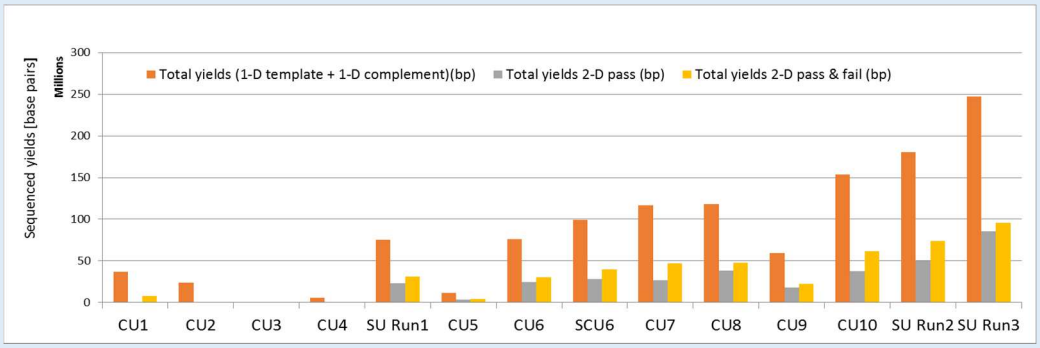


Figure 63. Improvement of MinION sequencing performance and yields over 6h of sequencing run time.

Legend: SU Spiked urine; CU clinical urine; SCU spiked clinical urine. Runs are shown in chronological order (see Table 34).

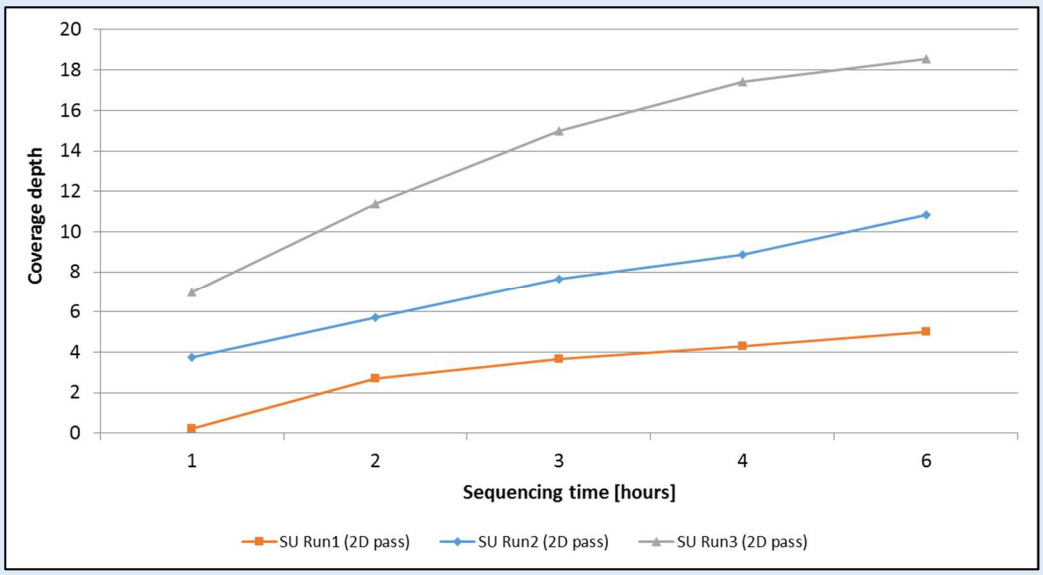


Figure 64. Timeline of coverage depth for successive runs with urine spiked with *E. coli* strain H141480453.

Run 4 cannot be included because it generated only 1-D reads, which are not suitable for automated ARMA software.

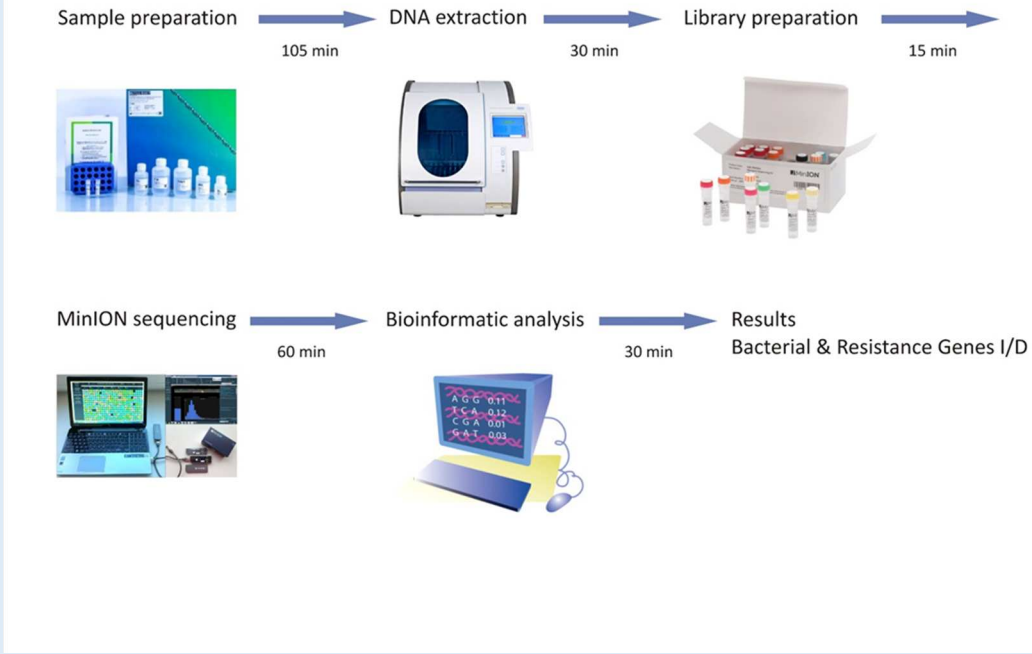


Figure 65. Timeframe of MinION sequencing with the 15-min library preparation kit used in Spiked Urine Run 4.

3.4.2 Bacterial identification

MinION analysis against BLAST and CARD databases was performed on 2-D sequence reads from the "pass" folder, containing only high quality data, for CUs 5, 6 and 7 and for the spiked urines, except Run 3 with *E. coli* H141480453, where WIMP/AMRA software was used (Figures 66, 67). For CUs 8, 9 and 10 we combined sequence reads from "pass" and "fail" folders to assess variation in quality between individual reads, again using BLAST and CARD. In context "fail" means "lower quality reads"; nevertheless, as we found here, these data remained useable.

In all cases, MinION correctly identified the pathogen present-variously *E. coli, Klebsiella pneumoniae* or *Enterobacter cloacae* (Table 39). Alignment to corresponding proteobacterial genomes exceeded 95%, except for CUs 8 and 10, at 93% and 90.3%, respectively. Alignment to human reads, which were discarded, was in the range 1.6-12.3%. Breadth of coverage was from 82.6-100%, being least for CU 5 (2.71x)- the first sample analysed successfully, and greatest, at 21.55x and 22.84x, for urine spiked with *E. coli* H141480453 (Run 2) and for CU 8, respectively (Table 39).

When in-built WIMP software was used, for Run 3 with urine spiked with *E. coli* H141480453, the organism was correctly identified within 15 min of real-time analysis. Outputs from this mode of analysis are illustrated in Figure 66 and 67.

An overview of Illumina sequencing data for the organisms grown from CUs 5-10 is presented in Table 40. The number of contigs was in a range 139-459 with maximum readlengths of 434525-1540822 bp and minimum readlength of 92 bp for all Clinical Urines. Alignments to Proteabacterial genomes was in a range 90.3-100%. Species identification was in perfect agreement with both MALDI-TOF and MinION sequencing directly from the urine.

Alignments to all *Proteobacterial* genomes from MinION and Illumina sequencing for Clinical Urines 5-10 and spiked urines with *E.coli* from Clinical Urine 6 and with *E. coli* H141480453 Run 1 and Run 2 are presented in Table D 1 in Appendix D.

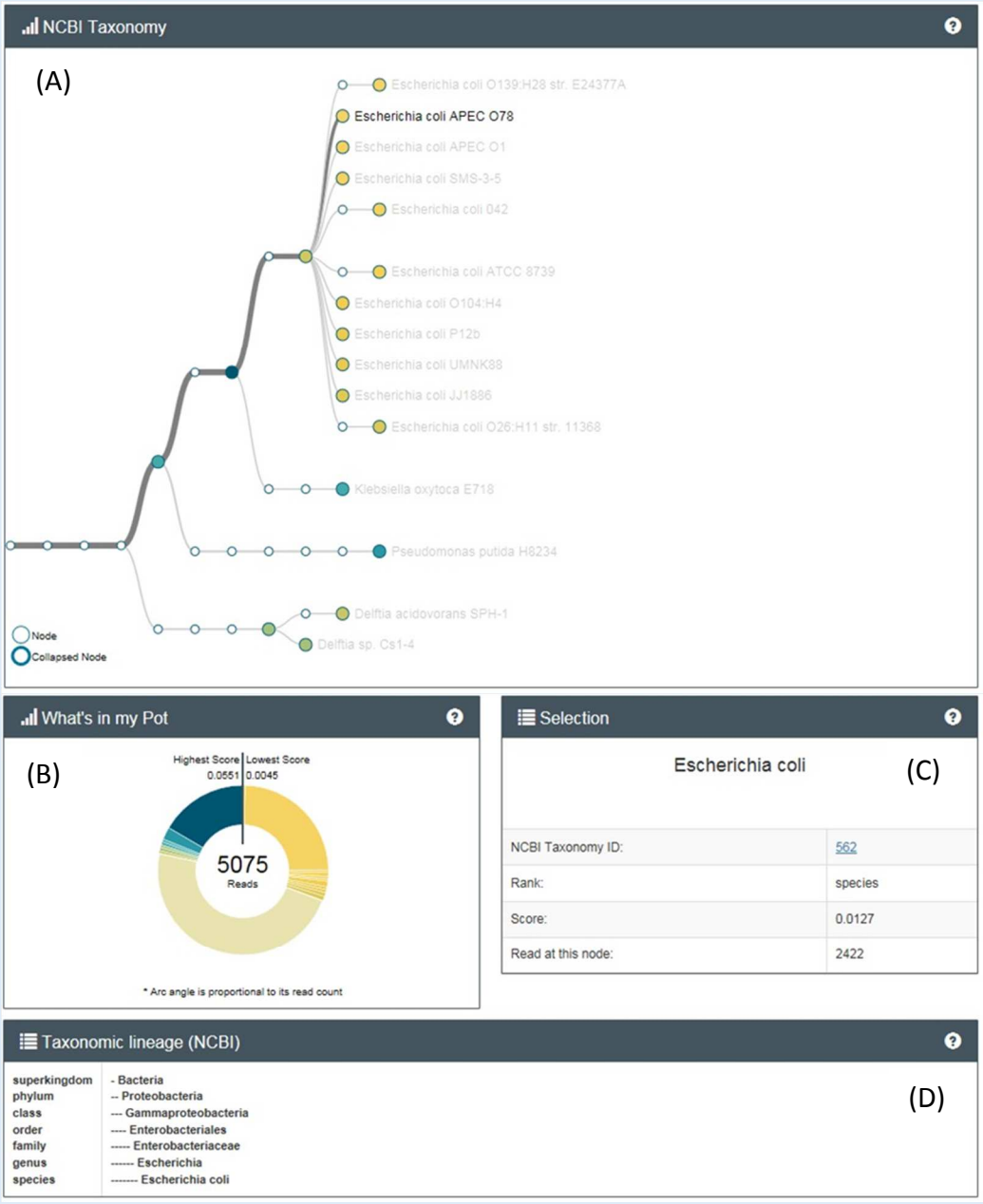


Figure 66. Output of the Metrichor WIMP application report for pathogen identification in MinION sequencing.

(A) NCBI Taxonomy: identifies the pathogens, in this case *E. coli*.

(B) Sample composition: shows the relative proportion of reads from the species found in sample and calculated confidence level.

(C) Selection: gives more details on the individual segment of sequence reads selected in the Sample composition section.

(D) Taxonomic linkage: contains details of the taxonomic classification for the individual organisms reported in the section B above.

(A)

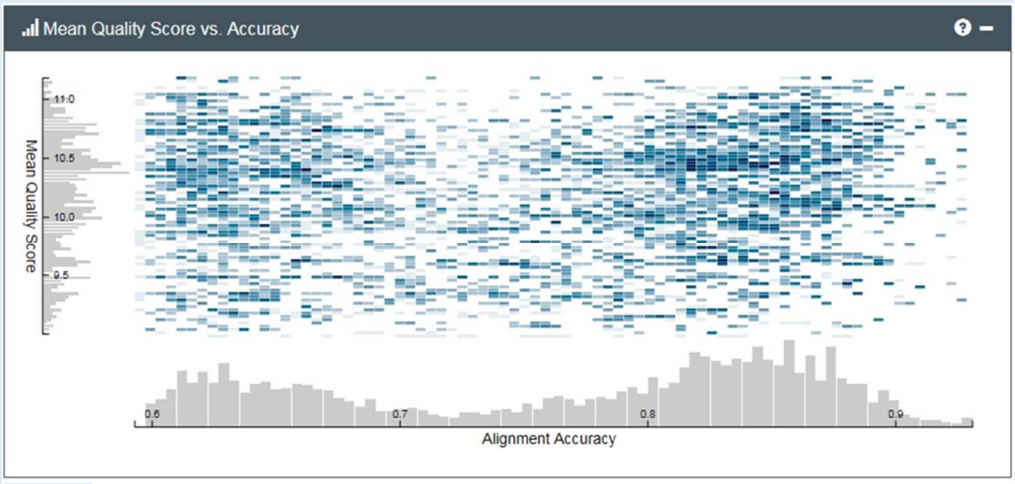
Alignments per ARO Molecule

Clear All Filters

Filter...

ARO Molecule	Alignments
Other	8018
elfamycin	613
sulfonamide	363
fluoroquinolone	340
macrolide	254
aminocoumarin antibiotic	224
lipopeptide antibiotic	189
tetracycline derivative	120

(B)



(C)

aadA5	112	112
dfrA12	**108**	**108**
aadA2	104	104
baeR	100	100
evgA	100	100
acrE	93	93
APH(3")-Ib	80	80

Identity Cluster	Gene	Total	Filtered
	AM040708.gene	**27**	**27**
	EF219134.3.gene12	27	27
	EU116440.1.gene1	27	27
	KJ363320.1.gene1	27	27

(D)

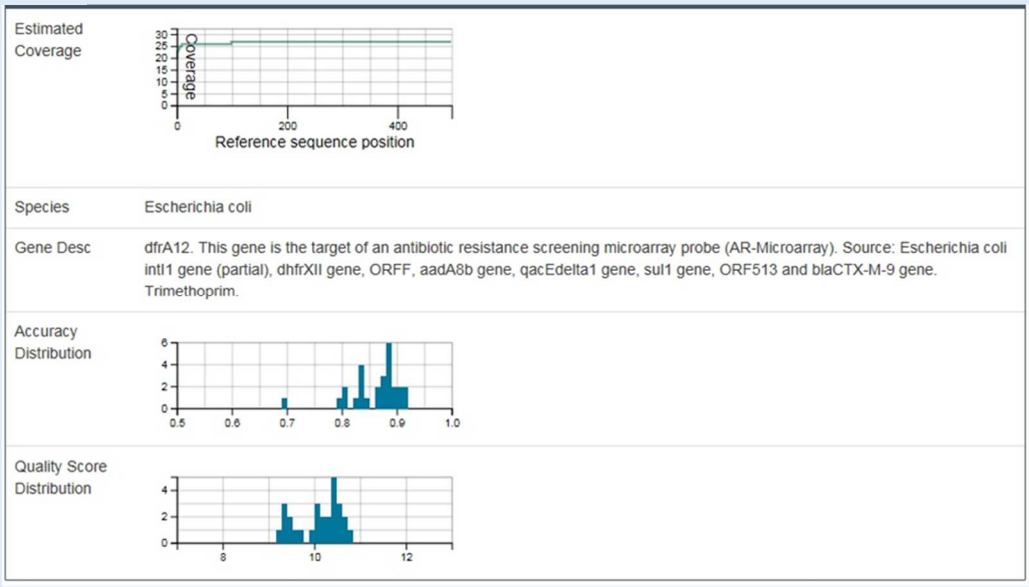


Figure 67. Output of the ARMA application for antibiotic resistance gene detection in MinION sequencing.

(A) Alignments per Antibiotic-Resistance Ontology (ARO): number of sequence reads with identity to genes associated with resistance to an antibiotic class.

(B) Alignments per ARO Molecule: mean quality score of MinION sequence reads versus alignment accuracy.

(C) Alignments per ARO Name: number of sequence reads associated with identified genes.

(D) Alignments per ARO Gene: gives more details on the coverage and accuracy of the gene matches to reference sequences.

Table 39. Pathogen identification using MinION sequencing for 6 clinical and spiked urines.

	Clinical Urine 5	**Clinical Urine 6**	**Urine spiked with *E. coli* from CU6**	**Clinical Urine 7**	**Clinical Urine 8**	**Clinical Urine 9**	**Clinical Urine 10**	**Urine spiked with *E. coli* H14148045 3 Run 1**	**Urine spiked with *E. coli* H14148045 3 Run 2**	**Urine spiked with *E. coli* H14148045 3 Run 3**
Reads used	2-D pass only	2-D pass only	2-D pass only	2-D pass only	2-D pass and fail	2-D pass and fail	2-D pass and fail	2-D pass only	2-D pass only	2-D pass only
% non-human DNA reads matching Gram-negative bacteria	76%	86%	83%	84%	81%	95%	85%	98%	89%	-
% DNA reads matching human	6.6%	8.5%	8.5%	8.1%	12.3%	1.7%	9.7%	1.6%	4.2%	-
Best species match to MinION sequence data	*K. pneumoniae* CG43	*E. coli* JJ1886	*E. coli* JJ1886	*E. coli* PMV-1	*E. coli* 536	*E. cloacae* NCTC 9394	*K. pneumoniae* CG43	*E. coli* APEC O78	*E. coli* KP-12	*E. coli* APEC O78
Best species match to Illumina sequence data	*K. pneumoniae* MGH 78578	*E. coli* JJ1886	*E. coli* JJ1886	*E. coli* IHE3034	*E. coli* 536	*E. cloacae* NCTC 9394	*K. pneumoniae* MGH 78578	*E. coli* ST410	*E. coli* ST410	*E. coli* ST410
% Breadth of coverage to best match organism	82.57%	99.59%	100%	92.19%	99.9%	86.25%	96.70%	95.13%	96.13%	-

	Clinical Urine 5	Clinical Urine 6	Urine spiked with *E. coli* from CU6	Clinical Urine 7	Clinical Urine 8	Clinical Urine 9	Clinical Urine 10	Urine spiked with *E. coli* H141480453 Run 1	Urine spiked with *E. coli* H141480453 Run 2	Urine spiked with *E. coli* H14148045 3 Run 3
Average depth of coverage *versus* best match organism	2.71 x	15.65 x	10.58 x	10.77 x	22.84 x	9.16 x	17.61 x	7.25 x	21.55 x	21.51 x
Run time (h)	25.5	23	14	17.5	36	26	35	30	48	7.5

CUs 1-4 are omitted as failures; limited data are shown in Table 22.

% non-human DNA reads matching Gram-negative bacteria and human genome were not calculated for Run 3 as the analysis was performed on automated WIMP and ARMA softwares.

Table 40. Overview of Illumina sequencing data for isolats grown from CUs 5-10.

	CU5	CU6	CU7	CU8	CU9	CU10
Number of contigs	139	232	197	459	171	167
Maximum readlength (bp)	1540833	654079	473756	557964	434512	461969
Minimum readlength (bp)	92	92	92	92	92	92
Mean readlength	39356	23809	26142	11541	27757	33692
Total bp	5470490	5523757	5149942	5297370	4746408	5626501
Percentage of Proteobacterial contigs	100%	100%	100%	99.3%	98.9%	90.3%
Total hits to Proteobacterial genome	2361 (92.7%)	3926 (94.4%)	4397 (93.2%)	4511 (85.9%)	5585 (96.5%)	2709 (94.2%)
Total hits to Firmicutes genome	156 (6.1%)	176 (4.2%)	249 (5.3%)	696 (13.3%)	176 (3%)	153 (5.3%)
Total hits to human genome	29 (1.2%)	58 (1.4%)	72 (1.5%)	44 (0.8%)	27 (0.5%)	15 (0.5%)

3.4.3 Resistance gene profiles for clinical urines

Acquired resistance genes were readily detectable in MinION outputs. Agreements to antibiogram and Illumina data for pathogen gene detection from Clinical Urines 5-10 and the spiked urines are summarized in Tables 41 and 42, respectively, and are detailed below. Agreements were generally good; among 55 acquired resistance genes detected by Illumina sequencing of cultivated bacteria, 51 were found by MinION directly from the urines; with 3 of 4 exceptions being in CU5, where coverage was the poorest. Limitations were: (i) MinION often flagged multiple gene variants (e.g. of bla_{TEM}, bla_{AmpC}, bla_{NDM}, $bla_{\text{CTX-M}}$) whereas Illumina definitively identified specific alleles, (ii) chromosomal *gyrA* and *parC* mutations associated with fluoroquinolone resistance were unreliably detectable from MinION data whereas they were detected by Illumina sequencing and (iii) plasmid and chromosomal *ampC*

were not discriminated and nor could the mode of *ampC* expression (basal, inducible or derepressed) be inferred.

Table 41 lists the acquired resistance genes found by MinION sequencing in the clinical urines in relation to Illumina results for the cultivated isolates and MICs by BSAC agar dilution. Relationships in these data are expanded in Section 3.4.4 below. Table 42 compares all MinION runs 1-4 and Illumina data for urines spiked with *E. coli* H141480453 in respect of acquired resistance genes only.

Table 42 shows a list of supplementary "resistance-related genes" found by MinION and Illumina sequencing. These are largely chromosomal genes associated with e.g. multidrug efflux pumps or outer membrane permeability (e.g. *mdt, ompF, acr, tolC*). They are present in all bacteria of these species and whether or not they relate to resistance depends on the level of expression. Relationships are notoriously complex. For example, overexpression of multidrug efflux pumps can arise either via mutations in their repressor genes or by activation of the global transcriptional regulators. We did not seek single nucleotide polymorphisms possibly affecting these function, owing to the low accuracy of MinION reads and the complexity of the interaction between different pumps and pump complements.

3.4.4 Agreement to phenotype and Illumina sequencing for Clinical urines

In this section the resistance genes found by MinION sequencing in CUs 5-10 are compared with the phenotypes of the isolates grown from these urines, and the resistance genes found in the organisms by Illumina sequencing (Table 41). In general, these datasets agreed well.

Clinical Urine 5 (K. pneumoniae)

MinION sequencing detected the extended-spectrum β-lactamase (ESBL) gene *bla*$_{CTX-M-15}$. This gene was also found by Illumina sequencing of the cultivated strain, which had an ESBL phenotype, with strong cephalosporin-clavulanate synergy. Both sequencing methods found *bla*$_{OXA-1}$, encoding an inhibitor-resistant penicillinase, congruent with the observed resistance to amoxicillin-clavulanate. Also in agreement, both methods found *dfrA14*, explaining observed trimethoprim resistance. MinION sequencing identified *bla*$_{SHV-32}$ whilst Illumina found *bla*$_{SHV-27}$ variant encoding ESBL and *bla*$_{LEN-12}$ (the *bla*$_{SHV}$- related) chromosomal β-lactamase genes *K. pneumonia,* along with *bla*$_{TEM-1}$ a penicillinase that would not further expand resistance in the presence of CTX-M-15. MinION did not find this *bla*$_{TEM-1}$ gene.

The strain was highly resistant to gentamicin and tobramycin, probably explained by presence of *aacC2.* It also had an *aac(6')-Ib-cr* gene, encoding a tobramycin- and amikacin-modifying enzyme, detected by both Illumina and MinION sequencing. The low amikacin MIC (2 mg/L) does not conflict with this: EUCAST advocates that isolates with *aac(6')-Ib* should be reported amikacin non-susceptible irrespective of MICs, as the enzyme inactivates amikacin. The presence of genes *strA* (detected by both methods) and *strB* (found only by Illumina) potentially explained observed streptomycin resistance.

Both MinION and Illumina sequencing detected *qnrB*, encoding a protein that protects DNA gyrase from fluoroquinolones. This protective protein only has a small effect on MIC, as does the *aac(6')-Ib-cr* gene also found by both sequencing approaches. Taken together, these findings agreed with the low-level ciprofloxacin resistance observed (2 mg/L). Chromosomal mutations in *gyrA* and *parC* as are usually associated with high-level resistance-were not detected by either sequencing method.

A sulphonamide gene (*sul2*) was found by both approaches while *fosA* gene, encoding fosfomycin resistance, was detected by Illumina only; phenotypic resistance to both sulphonamides and fosfomycin were not tested.

Clinical Urine 6 (E. coli)

In the case of Clinical Urine 6, infected with *E. coli*, MinION sequencing found $bla_{\text{CTX-M-15}}$ and $bla_{\text{OXA-1,}}$ with these genes also detected by Illumina sequencing and congruent with the phenotype, which implied ESBL production and included amoxicillin-clavulanate resistance a pattern typical of OXA-1, an inhibitor-resistant penicillinase. Both sequencing methods also indicated $bla_{\text{TEM-1}}$, a classical and extremely common penicillinase. Several plasmid- mediated *ampC* genes (bla_{CMY}, $bla_{\text{ACC-4}}$, $bla_{\text{MIR-9}}$, $bla_{\text{DHA-22}}$) were detected by MinION sequencing whereas Illumina indicated only $bla_{\text{CMY-113}}$ and $bla_{\text{MIR-14,}}$ albeit with identity below the 90% threshold. It is likely that all these *ampC* calls really corresponded to *E. coli* chromosomal *ampC,* as the cefoxitin MIC for the isolate was only 8 mg/L, whereas cefoxitin MICs for *E. coli* with acquired plasmid AmpC enzymes are mostly >64 mg/L (PHE data on file).

MinION sequencing found *aacC2* and *aac(6')-Ib-cr*, both confirmed by Illumina sequencing and in agreement with resistance to gentamicin and tobramycin and a raised amikacin MIC, though this remained in the clinically susceptible range (4 mg/mL). Both sequencing approaches also detected a streptomycin adenyltransferase (*aadA5*) determinant, but the organism was susceptible to streptomycin, implying that this was not expressed.

Detection of *dfrA17* by MinION sequencing was in agreement both with the Illumina result and with phenotypic resistance to trimethoprim. Detection of *aac(6')-Ib-cr* by both sequencing methods agreed with ciprofloxacin non-susceptibility, however the high MICs of ciprofloxacin (>8mg/mL) was more likely explained the presence of *gyrA/parC* mutations, which were found by Illumina sequencing but missed by MinION.

Both sequencing methods detected an acquired sulphonamide gene (*sul1*) but, as with CU5 and its isolate, phenotypic resistance to sulphonamides was not tested.

Clinical Urine 7 (E. coli)

Clinical Urine 7 again contained *E. coli*. MinION sequencing detected the ESBL gene *bla*$_{\text{CTX-M-15}}$, confirmed also by Illumina sequencing and agreeing with an ESBL phenotype. *bla*$_{\text{OXA}}$ was absent and the isolate was more susceptible than those from CUs 5 and 6 to penicillin β-lactamase inhibitor combinations. Both methods identified the penicillinase gene *bla*$_{\text{TEM-1}}$.

Phenotypic resistance to streptomycin agreed with detection of genes encoding nucleotydyltransferase variant *aadA1* and phosphotransferases (*strA* and *strB*) by both sequencing approaches also *aadA3,* which differs by several nucleotides from *aadA1*, by MinION only. Phenotypic resistance to trimethoprim agreed with detection of a *dfrA1* gene by both techniques.

An *ampC* gene (*bla*$_{\text{ACT-24}}$) was flagged by MinION, but not confirmed by Illumina sequencing. As with CU5, the low level cefoxitin MIC (4 mg/L) contraindicated plasmid *ampC*, and the MinION result probably reflected miscalling of chromosomal *ampC*. The ciprofloxacin MIC (0.25 mg/L) was slightly raised, and a single mutation in *gyrA* (83:S-L) was detected by Illumina only. Both sequencing approaches detected an acquired tetracycline gene *tet(A),* but phenotypic resistance to tetracycline was not tested, and EUCAST anyway has no breakpoints for Enterobacteriaceae in the case of this agent.

Clinical Urines 8 (E. coli) and 9 (E. cloacae)

The *E. coli* from CU8 was resistant to ampicillin, amoxicillin-clavulanate and cefoxitin (MICs, 16-64 mg/L), with diminished susceptibility to cefotaxime (MIC 1 mg/L). Cefotaxime-cloxacillin synergy implied AmpC activity, as did the strongly raised cefoxitin MIC (>64 mg/L). MinION flagged several acquired *ampC* genes (*bla*$_{\text{CMY}}$, *bla*$_{\text{ACC-4}}$, *bla*$_{\text{MIR-4}}$, *bla*$_{\text{DHA-6}}$, *bla*$_{\text{FOX-4}}$) but these were not confirmed by Illumina sequencing and this combined with the relatively low MIC of cefotaxime, mean that upregulation of chromosomal *ampC* is the more likely mechanism (in PHE's experience MICs of cefoxitin for *E. coli* with plasmid AmpC are usually ≥8 mg/mL). No genes were found for quinolone resistance, aminoglycoside-modifying enzymes and

trimethoprim dihydrofolate reductase resistance, which perfectly agreed with phenotypic susceptibility profile.

CU9 contained *E. cloacae,* with a cefotaxime MIC of 2 mg/L, reduced to 0.125 mg/L by cloxacillin, implying partial derepression of *ampC*, the commonest mode of oxyimino-cephalosporin resistance in this species. MinION flagged multiple acquired *ampC* genes (*bla*$_{CMY}$, *bla*$_{ACT-18, 24}$) and Illumina flagged *bla*$_{ACT-24}$, all probably reflecting misidentification of chromosomal *Enterobacter ampC*. No other acquired genes were found in either urine or isolate, agreeing with general susceptibility. MinION and Illumina also found *fosA2,* encoding a fosfamycin modifying enzyme, however fosfomycin susceptibility was not tested.

Clinical Urine 10 (K. pneumoniae)

Clinical Urine 10 was infected with a *K. pneumoniae* strain. MinION and Illumina sequencing detected *bla*$_{CTX-M-15}$ and *bla*$_{OXA-1}$, agreeing with an ESBL phenotype and amoxicillin-clavulanate resistance. Both sequencing approaches also found *bla*$_{TEM-1}$ and *bla*$_{SHV}$; Illumina identified the specific allele (*bla*$_{SHV-28}$), whilst MinION flagged multiple *bla*$_{SHV}$ variants. Resistance to gentamicin, tobramycin and amikacin (8-32 mg/L) corresponded to detection of *aac(6')-Ib-cr* and *aacC2* by both methods, with *aacA4* additionally found by MinION, perhaps owing to the lower identity cut off (80%) used with MinION compared to 90% for Illumina. Resistance to streptomycin agreed with detection of *strA* and *strB* by both methods and *aadA3* by MinION only. The plasmid-mediated quinolone resistance gene *qnrB* was identified by both methods along with *aac(6')-Ib-cr,* but high-level ciprofloxacin resistance (>8 mg/L) more likely reflected *gyrA* and *parC* mutations, found only by Illumina. Trimethoprim resistance agreed with detection of *dfrA14* by both techniques. Illumina again found *fosA2* gene, however fosfomycin susceptibility testing was not performed.

Spiked urines

Agreements between Illumina data and the four MinION sequencing runs using urine spiked with *E.coli* H141480453, which produced NDM-4 and OXA-181 carbapenemases, are summarized in Table 42. This organism was extremely drug resistant, with MICs >32 mg/L for ampicillin, amoxicillin-clavulanate, piperacillin-tazobactam, temocillin, cefotaxime, ceftazidime, cefepime, aztreonam, imipenem, meropenem, ertapenem, amikacin, gentamicin, tobramycin, ciprofloxacin, tetracycline, and trimethoprim, with susceptibility only to tigecycline (MIC 0.25 mg/L) and colistin (1 mg/L). Synergy was seen between EDTA and imipenem, reflecting metallo-β-lactamase inhibition, but not between cephalosporins and clavulanate or cloxacillin.

The four sets of MinION data, directly from spiked urine, closely matched Illumina sequencing for the cultivated isolate. Thus, the β-lactamase genes, bla_{TEM}, $bla_{\text{CTX-M-group-1}}$, bla_{OXA}, bla_{NDM} and bla_{CMY} were consistently identified, though with MinION flagging multiple matches within these families whereas Illumina identified precise alleles. Among aminoglycoside resistance determinants, *rmtB*, encoding a 16S rRNA methyltransferase, was consistently found by Illumina and MinION, as were *aacC2, aac(6')-Ib-cr* and *strA/*B; Illumina found all three of *aadA2*, *aadA3* and *aadA5* as did MinION run 3, with MinION runs 1 and 2 flagging only one or two of these. The genes *aadA2* and *aadA3* are closely related whilst *aadA5* differs considerably.

Trimethoprim resistance correlated with the presence of *dfrA12* and *dfrA17,* found both by Illumina and on all MinION runs except run 1, which had least coverage. Again, *aac(6')-Ib-cr* and *qnrS* variants were consistently flagged by both MinION and Illumina but are unlikely to have been the major contributors to the high level fluoroquinolone resistance (MIC >8 mg/mL), which is more likely explained by mutations in chromosomal *gyrA* and *parC,* which were reliable detected by Illumina only. The organism was sulphonamide resistant, and Illumina detected *sul1,* while all MinION runs found both *sul1* and *sul2*. Phenotypic tetracycline resistance agreed with Illumina sequencing, which found *tet (A)* and its regulator *tet(R*), though these were only detected in MinION Run 3. A *catB3* gene, congruent with chloramphenicol resistance was found by all MinION runs but not by Illumina, probably because, as

shown by review, this only had 69% homology to classical *catB3* well below the 90% thresholg used for Illumina.

3.4.5 Timeframe to detect genes

One hour of sequencing delivered 0.2x, 3.75x, and 6.96x coverage depth in spiked urine MinION Runs 1, 2 and 3, respectively (see Figure 64). To assess whether one hour was sufficient to detect all acquired resistance genes we reanalysed the MinION reads from these three spiked urine runs with *E. coli* H141480453 on the ARMA software and those of Run 4 with BLAST/CARD (its 1-D reads were unsuitable for ARMA). All the acquired resistance genes ultimately identified in runs 2, 3 and 4 were detectable within the first hour of sequencing, except for bla_{CMY} in run 2, and *strB* in run 4. An *ampC* gene (bla_{LAT-1}), was additionally identified in Run 4 (see Table 42). The lower coverage achieved at 1h for MinION run 1 (one of the first successful experiments) was insufficient to reliably detect $bla_{CMY,}$ *aadA2, aadA3, aac(6')-Ib-cr, strB* and *dfrA12* with high accuracy.

3.4.6 MinION sequencing for the same isolate from clinical urine and spiked urine

MinION sequencing of spiked urine with the *E. coli* isolate from Clinical Urine 6 detected the same acquired resistance genes found by Illumina sequencing of the cultivated isolate and MinION sequencing directly from CU6 (see Section 3.4.4) confirming that any bacteria and resistance genes in the urinary tract of the healthy urine-donor did not distort the results.

Table 41. Genes found by MinION sequencing for 6 Clinical Urines compared with antibiotic MICs and Illumina sequencing for cultivated isolates.

Urine and species	Method[a]	Penicillins and inhibitor combinations			Cephalosporins, monobactams and inhibitor combinations									Fluoro-quinolones	Aminoglycosides				Antifolate	Sulfonamide & Tetracycline
		AMP	AUG	PTZ	CTX	CTX-CLOX	CTX-CLAV	CAZ	CAZ-CLAV	CPM	CPM-CLAV	FOX	AZT	CIP	AMK	TOB	GEN	STR	TMP	SUL & TET
CU5 *K. pneumoniae*	MICs	>64	16	8	128	64	≤0.06	16	0.25	8	≤0.06	4	16	2	2	16	32	R	R	-
	MinION	*bla*$_{OXA-1}$			*bla*$_{CTX-M-15}$, *bla*$_{SHV-32}$									*qnrB* *aac(6')-Ib-cr*	*aac(6')-Ib-cr, aacC2, strA*				*dfrA14*	*sul2*
	Illumina	*bla*$_{TEM-1}$, *bla*$_{OXA-1}$			*bla*$_{CTX-M-15}$, *bla*$_{SHV-27}$, *bla*$_{LEN-12}$									*qnrB* *aac(6')-Ib-cr*	*aac(6')-Ib-cr, aacC2, strA, strB*				*dfrA14*	*sul2*
CU6 *E. coli*	MICs	>64	16	4	128	32	≤0.06	16	0.25	8	≤0.06	8	32	>8	4	16	16	S	R	-
	MinION	*bla*$_{TEM (mv*)}$, *bla*$_{OXA-1}$			*bla*$_{CTX-M\ gp1\ (15)}$, *ampC* (*bla*$_{CMY\ mv*}$, *bla*$_{ACC-4}$, *bla*$_{MIR-9}$, *bla*$_{DHA-22}$)									*aac(6')-Ib-cr*	*aac(6')-Ib-cr, aacC2,aadA5*				*dfrA17*	*sul1*
	Illumina	*bla*$_{TEM-1}$, *bla*$_{OXA-1}$			*bla*$_{CTX-M-15}$									*gyrA*[b] (83:SL; 87:D-N), *parC*[b] (80:S-I; 84:E-V), *aac(6')-Ib-cr*	*aac(6')-Ib-cr, aacC2,aadA5*				*dfrA17*	*sul1*
CU7 *E. coli*	MICs	>64	8	2	128	32	≤0.06	8	0.12	4	≤0.06	4	16	0.25	2	1	0.5	R	R	-
	MinION	*bla*$_{TEM (mv*)}$			*bla*$_{CTX-M\ gr1}$, *ampC* (*bla*$_{ACT-24}$)										*aadA1, aadA3, strA, strB*				*dfrA1*	*tetA/B/D/M*
	Ilumina	*bla*$_{TEM-1}$			*bla*$_{CTX-M-15}$									*gyrA*[b] (83:S-L)	*aadA1, strA, strB*				*dfrA1*	*tetA/R/D*

Urine and species	Method[a]	Penicillins and inhibitor combinations		Cephalosporins, monobactams and inhibitor combinations									Fluoro-quinolones	Aminoglycosides				Antifolate	Sulfonamide & Tetracycline
		AMP	AUG	PTZ	CTX	CTX-CLOX	CTX-CLAV	CAZ	CAZ-CLAV	CPM	CPM-CLAV	FOX	AZT	CIP	AMK	TOB	GEN	STR	TMP	SUL & TET
CU8 *E. coli*	MICs	64	32	4	1	≤0.12	0.25	0.5	0.5	≤0.12	0.12	>64	0.25	≤0.12	1	0.5	0.5	S	S	
	MinION					*ampC*(*bla*$_{CMY\ mv*}$, *bla*$_{ACC-4}$, *bla*$_{MIR-4}$, *bla*$_{DHA-6}$, *bla*$_{FOX4}$)														
	Illumina																			
CU9 *E. cloacae*	MICs	>64	64	4	2	≤0.12	2	1	1	≤0.12	0.12	>64	0.25	≤0.12	1	0.5	0.5	S	S	
	MinION					*ampC* (*bla*$_{CMY\ mv*}$,*bla*$_{ACT-18,\ 24}$)														
	Illumina					*ampC* (*bla*$_{ACT-24}$)														
CU10 *K. pneumoniae*	MICs	>64	32	>64	>256	256	0.125	128	1	64	≤0.06	16	>64	>8	8	>32	>32	R	R	
	MinION	*bla*$_{TEM\ (mv*)}$, *bla*$_{OXA-1}$			*bla*$_{CTX-M\ gr1}$, *bla*$_{SHV\ (mv*)}$,									*aac(6')-Ib-cr*, *qnrB*	*aac(6')-Ib-cr*, *aacA4*, *aacC2*, *aadA3*, *strA*, *strB*				*dfrA14*	
	Illumina	*bla*$_{TEM-1}$, *bla*$_{OXA-1}$			*bla*$_{CTX-M-15}$, *bla*$_{SHV-28}$, *bla*$_{LEN-12}$									*gyrA*[b] (83:S-I), *parC*[b] (80:S-I), *aac(6')-Ib-cr*, *qnrB*	*aac(6')-Ib-cr*, *aacC2*, *strA*, *strB*				*dfrA14*	

Legend: AMP, ampicillin; AUG, amoxicillin-clavulanic acid; AZT, aztreonam; PTZ, piperacillin-tazobactam; CTX, cefotaxime; CTX-CLAV, cefotaxime-clavulanic acid, CAZ, ceftazidime; CAZ-CLAV, ceftazidime-clavulanic acid; CPM, cefepime; CPM-CLAV, cefepime-clavulanic acid; FOX, cefoxitin; CIP, ciprofloxacin; AMP, amikacin, TOB- tobramycin; GEN, gentamicin; STREP, streptomycin; TRIM, trimethoprim. All β-lactamase inhibitors were used at 4 mg/L. **Red (R)**: resistant; **Yellow (I):** intermediate**; Green(S):** susceptible based on EUCAST criteria; **Orange**: acquired genes found only by Illumina; **Blue**: acquired gene families detected only by MinION; ***mv**: multiple (>5) different gene variants of this family were flagged.
[a] MICs are expressed as mg/L; MinION results are for the urine, tested directly; Illumina results are for the cultivated bacteria. Only relevant genes are listed.
[b] *gyrA* and *parC* were found in all clinical samples by both sequencing methods, they are only detailed when mutations were detected.

Table 42. Acquired resistance genes identified during four MinION runs for urine spiked with *E. coli* H141480453, compared with Illumina sequencing of the cultivated organism.

Genes	Illumina	MinION run 1 BLAST/CARD (run time = 30 h)	MinION run 2 BLAST/CARD (run time = 48 h)	MinION run 3 ARMA (run time= 1 h)	MinION run 4 BLAST/CARD (run time= 1 h)
β-Lactamase genes					
*bla*TEM	1[a]	1, mv[b]	1, mv[b]	1, mv[b]	1, mv[b]
*bla*CTX-M	group-1 (15)	group-1 (1, 3, 15, 52, 114)	group-1 (15, mv[b])	mv[b] not including bla*CTX-M-15*	mv[b] not including bla*CTX-M-15*
*bla*OXA	1, 181	31 (=1,30), 181	2, 7, 30, 232 (=181)	1, 181, mv[b]	181, mv[b] not including bla*OXA-1*
*bla*NDM	4	4, 6, 7	4, 5, 7, 12, 13	1	mv[b]
*bla*CMY	2	34, 45, 111	mv[b]	mv[b] not including bla*CMY-2*	mv[b] not including bla*CMY-2*
others	-	-	-	-	*bla*LAT-1
Aminoglycoside resistance genes					
aacC	*aacC2*	*aacC2*	*aacC2*	*aacC2*	*aacC2, aacC8*
aadA2, aadA3, aadA5	*aadA2, aadA3, aadA5*	*aadA2, aadA3*	*aadA5*	*aadA2, aadA3, aadA5,* mv[b]	mv[b] not including *aadA2,A3, A5*
rmtB	*rmtB*	*rmtB*	*rmtB*	*rmtB*	*rmtA*
aac(6')-Ib-cr	*aac(6')-Ib-cr*	*aac(6')-Ib-cr*	*aac(6')-Ib-cr*	*aac(6')-Ib-cr*	*aac(6')-Ib*
strA/B	*strA/B*	*strA/B*	*strA/B*	*strA/B*	*strA*
Quinolone resistance genes					
qnr	*qnrS1*	*qnrS3*	*qnrS3, qnrS7*	*qnrS1*	*qnrS*
aac(6')-Ib-cr	*aac(6')-Ib-cr*	*aac(6')-Ib-cr*	*aac(6')-Ib-cr*	*aac(6')-Ib-cr*	*aac(6')-Ib*
Trimethoprim resistance genes					
dfrA	*dfrA-12, dfrA-17*	not detected	*dfrA-12, dfrA-17*	*dfrA-12, dfrA-17*	*dfrA7 (A17) , A12, A21, A22*
Others					
cat	not detected	*catB3*	*catB3*	*catB3*	*catB3/B6*
sul	*sul1*	*sul1, sul2*	*sul1, sul2*	*sul1, sul2*	*sul1, sul2*
tet	*tetA, tetR*	*tetA, tetB, tetC*	*tetE*	*tetA, tetR*	*tetA, tetR*

Legend: [a] β-Lactamase gene variant detected; e.g. here '1' means *bla*TEM-1; [b] mv-multiple variants (> 5) flagged; **Grey**: additional acquired genes detected only by MinION, perhaps reflecting the lower (80% *vs.* 90%) identity threshold. Acquired resistance genes in MinION runs 1, 2 and 4 were sought using BLAST and CARD searches, whereas in run 3 they were sought using ARMA software.

Table 43. Additional genes mostly chromosomal potentially related to resistance found by MinION sequencing directly from clinical urines and Illumina sequencing of cultivated isolates.

Protein name	**Gene detected**	
	Urine 5	
	MinION	Illumina
β-Subunit of bacterial RNA polymerase	*rpoB*	*rpoB*
Dihydropteroate synthase	*folP*	-
Elongation factor EF-Tu2	*tufB*	*tufB*
Multidrug resistance protein	*mdtF*	-
DNA-binding transcriptional regulator	*cpxR*	-
Multidrug resistance protein D	*emrD*	*emrD*
Outer membrane channel protein	*tolC*	-
Outer membrane protein	*ompF*	-
Assembly protein	*asmA*	-
Multidrug efflux system protein	*acrA/B/D*	*acrR*
Protein	**Urine 6**	
	MinION	Illumina
β-Subunit of bacterial RNA polymerase	*rpoB*	*rpoB*
Dihydropteroate synthase	*folP*	*folP*
Macrolide 2'-phosphotransferase*	*mphA*	*mphA*
Multidrug efflux system protein	*mdfA*	-
Elongation factor EF-Tu2	*tufB*	-
Multidrug resistance protein	*emrA/B/E/R/Y*	*emrE/K/A/R/B/Y*
Multidrug resistance transporter protein	*mdtA/C/ D/E/L/F/G/H/K*	*mdtL/E/M/H/G/K/ C/F/O/D/B/A*
DNA-binding transcriptional regulator	*cpxA/R*	*cpxR/A*
Multidrug efflux system protein	*yjiO*	-
Outer membrane protein	*ompF*	*ompF*
Outer membrane channel protein	*tolC*	-
Assembly protein	*asmA*	-
Transcriptional regulator protein	*soxR*	*soxR*
Multiple antibiotic resistance protein	*marA*	*marA/R*
Transcriptional regulator protein	*crp*	*crp*
Transcriptional regulator protein	*gadX*	*gadX*
Sensory histidine kinase protein	*phoQ/P*	*phoQ/P*
Transcriptional response regulatory protein	*envR, Z*	*envR*

Protein name	**Gene detected**	
	Urine 6	
	MinION	Illumina
Transcriptional response regulatory protein	*baeR, baeS*	*baeR*
Sensory histidine kinase protein	*evgS*	*evgA/S*
Undecaprenyl pyrophosphate phosphatase protein	*uppP*	
Bifunctional polymyxin resistance protein	*yfbG*	*yfbG*
DNA-binding transcriptional dual regulator H-NS	*hns*	-
Undecaprenyl pyrophosphate phosphatase	*bacA*	-
Aminoglycoside/multidrug efflux system	*acrB/D/F/E*	acre/F/D
Protein	**Urine 7**	
	MinION	Illumina
β-Subunit of bacterial RNA polymerase	*rpoB*	*rpoB*
Dihydropteroate synthase	*folP*	*folP*
Macrolide 2'-phosphotransferase*	*mph(A)*	*mph(A)*
Elongation factor EF-Tu2	*tufB*	-
Multidrug efflux system protein	*mdfA*	-
Multidrug resistance protein	*emrE/K/Y*	*emrE/K/A/R/B/Y*
Multidrug resistance transporter protein	*mdtA/B/C/D/E/F/G/ K/L/M/H*	*mdtL/E/M/H/G/K/ C/F/O/D/A/B*
Multidrug resistance protein B	*ECs3548*	-
Multidrug resistance transporter protein	*omp36, ompF*	*ompF*
Outer membrane channel protein	*asmA*	
Cytoplasmic membrane lipoprotein	*acrF/E*	*acrF/D*
Outer membrane channel protein	*tolC*	*tolC*
DNA-binding transcriptional dual regulator	*soxS/R*	*soxR*
DNA-binding transcriptional regulator SoxS	*ECs5044*	-
DNA-binding transcriptional dual regulator	*marA*	*marR/A*
DNA-binding response regulator in two-component regulatory system with PhoQ	*phoP/Q*	*phoQ/P*
DNA-binding transcriptional dual regulator	*crp*	*crp*
DNA-binding transcriptional dual regulator	*gadX*	*gadX*
ATP-binding cassette (ABC) protein	*Lsa(C)*	-

Protein name	**Gene detected**	
	Urine 7	
	MinION	Illumina
Sensory histidine kinase in two-component regulatory system with CpxR.	*cpxA/R*	*cpxR/A*
Sensory histidine kinase in two-component regulatory system with OmpR.	*envR/Z*	*envR*
Sensory histidine kinase in two-component regulatory system with BaeR.	*baeS/R*	*baeS/R*
Sensory histidine kinase in two-component regulatory system with EvgA.	*evgS*	*evgA/S*
Undecaprenyl-diphosphatase	*uppP*	-
Bifunctional polymyxin resistance protein ArnA	*yfbG*	-
Aminoglycoside/multidrug efflux system	*acrB/D*	*acrE*
Protein	**Urine 8**	
	MinION	Illumina
β-Subunit of bacterial RNA polymerase	*rpoB*	*rpoB*
Macrolide 2'-phosphotransferase*	*mph(A)*	
Elongation factor EF-Tu2	*tufP*	
Multidrug resistance protein	*emrA/B/K/Y*	*emrE/K/A/R/B/Y*
Multidrug resistance transporter protein	*mdtA/B/C/D/E/F/G/ K/L/H*	*mdtL/E/H/G/K/C/ F/O/D/P/B/A*
Multidrug resistance transporter protein	*ompF*	*ompF*
Aminoglycoside/multidrug efflux system	*acrE /D*	*acrE /F/D*
Outer membrane channel protein	*tolC*	*tolC*
DNA-binding transcriptional dual regulator	*soxR*	*soxR*
DNA-binding transcriptional dual regulator	*marA*	*marR/A*
DNA-binding response regulator in two-component regulatory system with PhoQ	*phoQ/P*	*phoQ/P*
DNA-binding transcriptional dual regulator	*crp*	*crp*
DNA-binding transcriptional dual regulator	*gadX*	*gadX*

Protein name	**Gene detected**	
	Urine 8	
	MinION	Illumina
Sensory histidine kinase in two-component regulatory system with CpxR.	*cpxA/R*	*cpxR/A*
Sensory histidine kinase in two-component regulatory system with OmpR.	*envR/Z*	*envR*
Sensory histidine kinase in two-component regulatory system with BaeR.	*baeS/R*	*baeR*
Sensory histidine kinase in two-component regulatory system with EvgA.	*evgA/S*	*evgA/S*
Bifunctional polymyxin resistance protein ArnA	*yfbG*	-
DNA-binding protein H-NS	*hns*	-
Protein	**Urine 9**	
	MinION	Illumina
β-Subunit of bacterial RNA polymerase	*rpoB*	*rpoB*
Multidrug resistance protein	*mdtB/C*	-
Multidrug resistance protein	*emrB/D/R*	-
Sensory histidine kinase in two-component regulatory system with BaeR.	*baeR*	-
Multidrug resistance transporter protein	*ompF*	-
Sensory histidine kinase in two-component regulatory system with CpxR.	*cpxR, cpxA*	-
DNA-binding response regulator in two-component regulatory system with PhoQ	*phoQ*	-
Aminoglycoside/multidrug efflux system	*acrD*	-
Protein	**Urine 10**	
	MinION	Illumina
β-subunit of bacterial RNA polymerase	-	*rpoB*
Multidrug resistance protein	*emrD*	-
Multidrug efflux system protein	*mdfA*	-
Aminoglycoside/multidrug efflux system	*acrR*	*acrR*

Legend: *Plasmid-or integron-mediated but affecting agent with little anti-Gram-negative activity.

Chapter 4

DICSUSSION

Culture-based methods take *c*. 24-72 h to identify bacteria and to determinate which antibiotics can be used for definitive treatment. During this period patients receive empirical antibiotics, but these might be inadequate or disproportionate. Fast, precise identification of bacteria and their resistances has the potential to resolve this situation, ensuring that (i) appropriate treatment can be prescribed at an early stage and (ii) our diminishing antibiotic reserves are better managed.

The main purpose of this study was to develop and evaluate rapid diagnostics to identify pathogens and resistances directly from urine samples for high-risk urosepsis patients. Urine was investigated because UTIs are the source of many septic episodes, especially with *E. coli*, which is now the commonest agent of bacteraemia in the UK. Moreover, urine is a relatively 'easy' specimen to test because the collection process is not invasive, and infected urine contains high numbers of bacteria compared to other biological samples such as blood, tissues, cerebrospinal fluid, where bacteria are hugely outnumbered by human cells, making analysis more difficult. Unlike respiratory secretions, urines rarely contain large numbers of commensal flora. Methodologies developed for urine coupled with human cell depletion nevertheless may transfer to other specimens and provide a swifter microbiological analyses in urgent cases than currently possible.

There are several methods that could potentially be used to accelerate diagnostics. In our study we evaluated two approaches: (i) MALDI-TOF for bacterial identification and β-lactamase differentiation combined with multiplex tandem real-time PCR for antibiotic resistance gene detection, and (ii) MinION sequencing to simultaneously identify both pathogens and their resistances.

In the first approach pathogen identification from urine could alternatively be performed by PCR, as evaluated in some studies (Lehmann *et al.*, 2011, Cybulski *et al.*, 2013). PCR has greater sensitivity (3-100 cfu/mL) (Lehmann *et al.*, 2011) than mass spectrometry (>10^5 cfu/mL) but is limited to detecting those species sought by the

primers or probes, whereas MALDI-TOF can identify the full range of pathogens. On this basis, we preferred mass spectrometry for bacterial identification and optimized a procedure for sample preparation directly from urine.

Multiplex-tandem PCR was preferred over other PCR methodologies to seek common resistance genes among Enterobacteriaceae because (i) it seeks to establish conditions whereby amplicons of interest are first enriched by limited PCR and then amplified, whereas simpler multiplex PCR creates condition where less-efficiently amplied targets may be missed. Three different interations of the PCR assay were explored.

In the second approach, the sequencing-based nanopore technology aimed to provide a comprehensive method to identify bacteria and their acquired resistance genes in a timeframe similar (or even faster) than the MALDI-TOF/PCR approach. Although Ion-Torrent sequencing has been used directly from urine (Hasman *et al.*, 2014), this is slower and more laborious than the nanopore method explored here.

As background, an understanding of the local epidemiology of UTI is very important. Consequently, an epidemiological surveillance of urinary pathogens was performed.

Different urine samples and numbers of urine samples were used for evaluation of each of the approaches. Sample collection reflected a deliberate strategy aiming to seeing to represent (i) pathogen diversity, (ii) β-lactamase producing strains and (iii) various antibiotic resistance genes. Cost factors caused that fewer sample could be analyzed by MinION, MALDI-TOF and PCR. Throughout, the aim was to demonstrate proof of concept with appropriate sample selection, not to directly compare (with the same sample) the performance of the difference approaches considered.

4.1 Epidemiological study

The literature suggests that approximately 60-80% of urine samples sent to clinical laboratories in Europe for UTI analysis are negative for infection (Pieretti *et al.*, 2010; Falbo *et al.*, 2012; De Rosa *et al.*, 2010). This view was supported by our

epidemiological study, where 61.6% (July) and 59.3% (November) of all submitted urines were negative for infection based on automated microscopy screening (Iris) or culture analysis. High numbers of negative results may reflect i) routine screening of pregnant women and/or patients with vaginal symptoms, seeking to rule out UTIs, (ii) routine culture on urines from children, neonates, patients in ITU and high-risk groups such as patients with immunodeficiency or those receiving chemotherapy or corticosteroids, iii) infections caused by fastidious pathogens difficult to cultivate on standard medium, iv) patients with small numbers of bacteria that do not meet the criteria for significant infection ($>10^5$ cfu/mL) or v) UTI being present but antibiotic therapy being implemented before culture was performed, preventing recovery of the bacteria.

One of the major challenges of analysis is the poor linkage of microbiology and clinical data. Here, as in many studies, there was no information on the laboratory system on whether the submitted urine specimens came from patients with symptomatic upper or lower UTIs, asymptomatic bacteriuria, recurrent UTIs, and/or whether they were routine screens. The use (or not) of point-of-care tests e.g. dipsticks in clinical settings and by GPs was also unknown and there were no data on whether or not empirical antibiotic therapy had been implemented based on clinical symptoms.

The recent PHE ESPAUR report noted that nationally 52.4% of positive urine cultures were from general practices in 2014 (PHE, 2016a). In our study, most (>67%) of all the positive urine cultures were from GP patients in all age group. Negative cultures dominated in more specialized locations i.e. Hospital In- and Out- patients, Admission Units, probably reflecting greater routine screening of new patients and monitoring of treatment in these care settings. The number of positive urine cultures for these latter units grew slowly with the age group. The reason for this increase might be associated with screening elderly admission with non-specific symptoms or screening catheterize patients in order to prevent complicated UTIs.

4.1.1 Aetiology of UTIs

It is generally accepted that the incidence of urinary infection varies with sex, age and the presence of underlying genitourinary abnormalities. Women are more prone to UTI than men; this is associated with the anatomy of urinary system, where the female urethra is much shorter and closer to the anus. This sex distribution was confirmed in our study: in both months 67% of all submitted urines were from women. Age is the second factor that has an impact on the risk of developing UTI. Unsurprisingly, for both genders, the highest numbers of urine examinations were performed for the elderly group, aged 66-85 years, who also accounted for the highest proportion of positives. There are many factors highlighted in the introduction (see Section 1.4) that tend to increase the risk of complicated UTIs in elderly populations, although uncomplicated UTIs, especially cystitis, mostly occur in the community among women aged <50 years old.

UTIs also occur in hospitals, and a Pan-European study reported a general incidence of hospital-acquired UTIs at 10.65 per 1000 patient bed days (Bouza *et al.*, 2001) to which must be added patients who are admitted with a complicated or ascending UTI. In the UK, these latter patients would ordinarily have urine taken in the Admission Unit or Accident and Emergency. Although the prevalence of nosocomial-acquired UTIs varies widely between countries and hospitals, one study performed by Bjerklund Johansen *et al.* (2007) suggests an overall prevalence of hospital-acquired UTIs of around 10% in Europe and 14% in Asia (Bjerklund Johansen *et al.*, 2007). In our study, approximately 14% of the urines submitted for examination were from Hospital In-patients in both months with the proportion of urines submitted for Admission Units was slightly higher in November (12.5%) than in July (11.1%). The differences between the two months might reflect general “winter pressure” with non-specific signs and symptoms of infections in November. Although most “winter pressure” patients have more admissions because of respiratory tract infection, urines are likely to be taken to exclude alternative origins of infection.

4.1.2 Epidemiology of UTIs

The ARESC study (Antimicrobial Resistance Epidemiological Survey on Cystitis) carried out in nine European countries reported that *E. coli* was responsible for 76.7% of uncomplicated UTIs, ranging from 68.1% in Austria to 83.8% in France (Schito *et al.*, 2009). Similar proportions of *E. coli* were found here- 68% in both July and November. Overall, *E. coli* predominant in all age ranges, and in all location types. Distributions of other etiological agents causing UTIs were similar to found by other studies (Farrell *et al.*, 2003; Linhares, 2013; Lu *et al.*, 2012).

4.1.3 Antibiotic resistance

Among *E. coli*, resistance rates to antibiotics used to treat UTIs vary widely between continents, countries, and also between community and hospital-acquired urinary infections. A UK study of the antimicrobial susceptibility of *E. coli* from primary and secondary care showed the highest rates of resistance to trimethoprim (range: 30.3% to 41.8%) and ampicillin/amoxicillin (range: 55%-57.7%) (PHW, 2016; HPS, 2016; PHE, 2016a). In our study we observed similarly high resistance rates to these agents, through with significant fluctuations according to time and locations for both antibiotics e.g. 54.3% resistance in July *vs*. 61.3% resistance in November for amoxicillin, and 45% in July *vs.* 34.7% in November for trimethoprim in Hospital Inpatients, and 35.5% resisiance in July *vs.* 17.9% resistance in November for trimethoprim in Hospital Outpatients. This may indicate seasonal fluctuation in strains or chance, but does suggest that the appropriateness of prescribing may fluctuate.

The high resistance rates for trimethoprim in the UK led to review of treatment guidelines for UTIs in 2014 and to substitution of nitrofurantoin for trimethoprim as the first line empirical therapy for uncomplicated cystitis. This new recommendation caused trimethoprim use to fall by 14.5% between 2014/15 (PHE, 2016a). Although the nitrofurantoin reststance rate remained stable at 3-5% for *E. coli* -and slighly higher, at 10-20%, for other coliform species- in both community and nosocomial-

acquired UTIs in the UK in the years 2005-2014, trimethoprim remains a better oral agent against susceptible strains than nitrofurantoin for several reasons:

Firstly, trimethoprim can be used to treat both cystitis and incipient ascending infection, if the pathogen is susceptible, whilst nitrofurantoin attains low concentration in renal tissue and the bloodstream and therefore is inactive for incipient pyelonephritis or bacteraemia (Oplinger & Andrews, 2013).

Secondly, effective antibiotic therapy takes 3 days for trimethoprim, compared to 5-7 days of nitrofurantoin (Gupta *et al.*, 2011).

Thirdly, in the absence of acquired resistance, trimethoprim is active against both Enterobacteriaceae and Gram-positive bacteria, though its use for enteroccocal UTIs is controversial as enterococci, despite low MICs (Gordon *et al.*, 2003; Hoban *et al.*, 2003) may be resistant because these bacteria can absorb and use the exogenous folic acid present in urine (Wisell *et al.*, 2008). Trimethoprim is more active against UTIs caused by *Staphylococcus* spp. and *Streptococcus* spp. whilst nitrofurantoin may not be effective in the alkaline urine produced by urease-producing bacteria e.g. *S. saprophyticus*.

Fourthly, some bacteria e.g. *Proteus* spp., *Morganella morganii*, *Providencia* spp. and *Serratia* spp. are inherent resistant to nitrofurantoin (Livermore *et al.*, 2001).

Fifthly, trimethoprim is mostly well-tolerated by patients of all ages whilst the use of nitrofurantoin in moderate renal impairment or in long-term/repeated courses may be associated with pulmonary toxicity (Williams & Triller, 2006).

Lastly, nitrofurantoin requires good renal function to achieve adequate concentration in the urine and is only acceptable if eGFR is >45 mL/min, whereas there is no such need for trimethoprim (MHRA, 2014)

Treatment of upper UTIs eg. pyelonephritis in the community is a challenge, as most oral drugs suitable for cystitis such as nitrofurantoin, pivmecillinam and fosfomycin are ineffective. The growing spread of Enterobacteriaceae producing β-lactamases including ESBLs (mostly CTX-M-15 in *E. coli* ST131) or mutationally derepressed of AmpC types in e.g. *Enterobacter* spp. and *Citrobacter freundii* (Meier

et al., 2011) in the community or nosocomial UTIs complicates treatmens. Most ESBL producers are resistant against many agents i.e. trimethoprim, quinolones, oral cephalosporins and more variably, co-amoxiclav (Pallett & Hand, 2010). In this case patients require hospitalization for intravenous carbapenems, piperacilin/tazobactam (contingent on susceptibility data) and aminoglycosides, again contingent on susceptibility data, even when they ordinarily could be managed in the community. New combination of cephalosporins with β-lactamase inhibitors: i.e. ceftazidime/avibactam and ceftolozane/tazobactam have proved to be active against Enterobacteriaceae producing ESBLs including TEM, SHV, CTX-M types, also AmpC producers in the case of ceftazidime/avibactam (Coleman *et al.*, 2014; Flamm *et al.*, 2014; Sader *et al.*, 2015; Wagenlehner *et al.*, 2015; Liscio *et al.*, 2015), however they are expensive, meaning that swift recognizion of those cases that retain susceptibility to older agents might be advantages both in term of consuming broad spectrum antibiotics but also cost-effective.

4.1.4 Seasonal variations in UTIs

Seasonal variations in urinary infection rates for Gram-negative bacteria have not been widely investigated or described in literature. However, several studies stress that bloodstream infection rates for *E. coli*, *E. cloacae* and *K. pneumoniae* increase significantly during the summer period (Chazan *et al.*, 2011; Anderson, 1983; Al-Hasan *et al.*, 2009; Eber *et al.*, 2011), and for *E. coli* at least, most of these bacteraemia are secondary to UTIs. Seasonal variations for UTIs and contingent bacteraemia might be associated with several factors: (i) meteorological parameters (high temperature, humidity) modulating host vulnerability e.g. via dehydratation and sweating, (Falagas *et al.*, 2009), (ii) environmental factors (water and food contamination), (iii) seasonal fluctuation in host immune function (Freeman *et al.*, 2009) or (iv) seasonal changes in bacterial virulence strain prevalence and antibiotic resistances (Freeman *et al.*, 2009).

Higher temperatures during the summer may promote the growth of bacteria colonizing the skin and indwelling devices e.g. catheters; also there may be increased

contamination of food and water sources with pathogenic *E. coli* during the summer (Perencevich *et al.*, 2008; Alcorn *et al.*, 2013). Hot weather may also induce dehydration, leading to decreased diuresis, more concentrated urine and less frequent voiding. It might also lead to moisture in the perineum, facilitating bacterial transfer from the rectum to the urethra (Anderson, 1983). Some authors are more skeptical and Deeny *et al*. (2015) suggested that meteorological factors i.e. higher temperatures and humidity during summer period rarely have an impact on seasonal infections in the UK, especially in the (colder) north of England (Deeny *et al.*, 2015).

A high incidence of community-acquired UTIs in women, caused by *E. coli*, could also be explained by increased sexual activity during the summer months (Freeman *et al.*, 2009).

In our epidemiological study we did not analyze the patient comorbidities on the seasonality of UTIs. However, we observed a slight increases in emergency admission of patients aged >65y in November, boosting the number of positive cultures.

E. coli strains vary widely in their propensity to cause disease. Enterotoxigenic and enterohemorrhagic *E. coli* strains causing diarrhoea seldom achieve asymptomatic gut colonization whilst *E. coli* strains causing extraintestinal infections (ExPEC) colonize the intestinal tract, and have virulence genes adapting them to do so (Johnson, 2003). Certain ExPEC clonal groups i.g. ST73, ST131, ST95, ST69, ST12 account for a large and growing proportion of *E. coli* UTIs in humans (Giedraitienė *et al.*, 2017; Rogers *et al.*, 2011; Johnson *et al.*, 2009; Day *et al.*, 2016).

In analyzing the seasonality of urinary infections it would be useful to determine whether or not the climatic and/or environmental factors influence the occurrence only of primary uncomplicated UTI but also complicated and ascending UTIs (which might explain the summer peak of bacteraemia). One study in South Korea showed that acute pyelonephritis occurs more commonly during the summer season in all age groups (Ki *et al.*, 2004). Taking into account that (i) the majority of hospital-acquired UTIs in the UK are associated with long-term catheters, and (ii) the catheter-UTIs account for 8% of all nosocomial bacteraemias (SMCAPG, 2014) it would be interesting also to perform a study of prevalence of catheter-acquired UTIs and contingent bacteraemias in the summer and winter periods and link these to possible contributory factors.

We observed a slightly greater number of urine samples submitted for examination in July (n=9558) than in November (n=8991), again supporting the view of seasonality of UTI. Anderson (1983) also noted a higher number of GP submissions in the summer period (Anderson, 1983). However, a caution is that our epidemiological surveillance was performed in one year, and for only two months. To fully test the seasonality of UTIs in Norfolk the study needs to be repeated analyzing the data for a minimum of two years. Explaining seasonal variations of UTI could guide infection prevention interventions.

4.1.5 Pathogen distribution and patients group

Although the overall proportions of pathogens isolated was similar between the two months studied, we observed differences in the pathogen distribution for female population only, between July and November period. Ipe *et al* (2013) also found that some bacteria disproportionately affect different patient populations, particularly in continuously asymptomatic bacteriuria. For example, *E. coli* was less prevalent among healthy men and patients with indwelling catheters whilst *Enterococcus* spp. were cultured from almost a quarter of bacteriuric healthy men, but only 3–4% of bacteriuric pregnant women (Ipe *et al.*, 2013). In our study we observed that some pathogens more frequently isolated in particular age groups of women than men. One example is *S. saprophyticus,* which was detected more often in the female population aged 16-45 than in men, as also found in another study (Jordan *et al.*, 1980). This pathogen also occurred twice as often in November than in July. Finally group B streptococci was mostly seen in women in the age groups 16-45y and 45-65y, similar to the situation in other studies (Edwards & Baker, 2005).

Enterococcus spp. were the second commonest pathogens after *E. coli* among the female population aged <15 y for both months. Although there was a paucity of clinical details, this might be associated with anatomical abnormalities, recurrences caused by inappropriate empiric antibiotic therapy, renal scarring, and vesicoureteral reflux (Bitsori *et al.*, 2005; Marcus *et al.*, 2012). We also noted that *Enterococcus* spp. were isolated more frequently in July than in November in adults and elderly populations (46-65y and 66-85y) for both genders. *Staphyloccocus aureus* rarely

cause bacteriuria in healthy adults (Al Mohajer & Darouiche, 2012), agreeing with our results (<0.7%); however this pathogen is relatively more prevalent among pregnant women (Imade *et al.*, 2010), diabetic patients (Nicolle, 2006), and community-dwelling elderly men (Woodford *et al.*, 2011).

Among Gram-negative species *Pseudomonas* spp. and Proteeae were the third and fourth most frequent pathogens isolated from elderly populations for both genders; again these species are linked to indwelling catheters and instrumentalisation (Hooton *et al.*, 2010; Nicolle, 2014; Nicolle, 2012).

4.1.6 Empirical treatment

Understanding local resistance rates in both GP and hospital settings is critical to deciding when to change guideline recomendations for empirical therapy, including in UTIs. The risks of treatment failure evidently are important to the choice of treatment options. A threshold of 20% resistance in uncomplicated UTIs and a lower cut off 5-10% resistance in complicated UTIs could be used as an indicator to change empirical treatment.

Monitoring of antibiotic use and the spread of resistances in the communities and hospitals is necessary for effective antimicrobial stewardship. In 2014 the majority of antibiotics in England were prescribed for GP patients (74%) followed by hospital in-patients (11%) and hospital out-patients (7%) (PHE, 2016a). Different studies report discordant results for the relationship between antibiotic use and antibiotic resistance in urinary *E. coli* (Bartoloni *et al.*, 2004; Colgan *et al.*, 2008). Nevertheless a strong relationship between increases in trimethoprim prescription rates resistance was showed by (Vellinga *et al.*, 2012; Metlay *et al.*, 2003).

In the UK, national prescribing data are available only at general practice level (Donnan *et al.*, 2004). However, the relation between antibiotic resistance and individual patients' level should be taken into account when antibiotic therapy is adjusted. Donnan *et al*. (2004) showed that resistance to trimethoprim was significantly associated with patients' age, gender and -critically- individual level exposure to trimethoprim, and other antibiotics. Steinke *et al.* (2001) stressed that

trimethoprim resistance was independently associated with exposure not only to trimethoprim but also other antibiotics e.g. ciprofloxacin (Steinke *et al.*, 2001). Previous travel and migrations from the countries where resistance rate is high i.e. Middle East, south Asia, is also a risk factor for acquired multidrug resistant bacteria (Hassing *et al.*, 2015). One study in Birmingham showed significant difference between carriage CTX-M-15 *E. coli* producing strain in those with European group (8.1%) *vs.* Middle East/South Asian (22.8%) (Wickramasinghe *et al.*, 2012). These observations underscore the overall challenge for empiric antibiotic therapy and how risk of inadequate therapy can vary among individuals and groups.

4.1.7 Where rapid diagnostic might help

The present survey indicates several places and settings where a rapid diagnostic might be useful.

Firstly, trimethoprim has been largely abandoned in UTIs owing to high resistance rates. Nonetheless, approximately 65% of Enterobacteriaceae responsible for UTIs remains susceptible to trimethoprim. Knowing that *E. coli* predominant in community and hospital acquired UTIs, resistance rates for trimethoprim is high but trimethoprim is a better agent for UTIs than nitrofurantoin. It is potentially valuable to develop and implement rapid and automated point-of-care methods for simultaneously *E. coli* and trimethoprim-resistance profiling in the community. Much the same points could be made in the case of qluoroquinolones. Such a test may improve prognosis, management of UTIs and might also save patient hospitalizations and diminishing the use of antibiotics i.e. IV carbapenems or piperacillin/tazobactam that might better be reserved to treat severe infections, whether or not of urinary orgin.

Secondly, since the number of emergency admission for complicated UTI for the population aged >66 years old increased by 100% in the period 2003-2012 in the UK (PHE, 2015), and the number of bloodstream infections due to *E. coli* (which mostly have a urinary origin) has been increasing year on year with an estimated mortality rate 18.2% (Deeny *et al.*, 2015) this indicates a potential location (i.e. Acute Medical

Units) and patients with suspected bacteremia where a rapid diagnostic might usefully be implemented.

Given the importance of *E. coli* in UTIs and, among these importance the global spread of clonal group ST131, which is often more resistant than other STs (Day *et al.*, 2016), indicates it might be beneficial to rapidly seek ST131 in urine samples, e.g. by a specific PCR.

In all these cases the swift detection of pathogen and resistance has the potential to guide early therapy, long before culture results would ordinarly be obtained.

Thirdly, short-term fluctuations in susceptibility rates, as seen here for amoxicillin and trimethoprim in both months for Hospital Inpatients and Outpatients, show the weakness to particular units of empitical therapy guidelines whose appropriateness likewise may vary from month to month. It should also be stress that the higher the resistance rate, the more likely it is that any empirical therapy will prove inappropriate. In context the resistance rates for Enterobacteriaceae and non-fermenters *Pseudomonas* spp. isolated from UTIs in the East Anglia are generally lower than in North Western England and in London. Understanding the local epidemiology and resistance rates may help to identify particular locations and patient group where rapid diagnostics- whether broad-based or specifically seeking trimethoprim resistance might usefully be implemented.

4.2 Rapid bacterial identification directly from urine samples using MALDI-TOF

MALDI-TOF has been widely adopted as a rapid and powerful diagnostic tool in clinical microbiology laboratories. In comparison with conventional methods, e.g. biochemical tests, microscopy and culture, MALDI-TOF significantly shortens the time required for identification. Numerous studies have described the power of MALDI-TOF for bacterial and fungal identification to species level using colonies taken from culture plates. Identifications are obtained in few minutes without a long sample preparation procedure (Bizzini *et al.*, 2010; Marklein *et al.*, 2009; Wang *et al.*, 2014a). MALDI-TOF has also been successfully used for direct identification without sub-

culture from the growth in blood culture bottles, and directly from urine (Hong *et al.*, 2014; Ferreira *et al.*, 2011; Ferreira *et al.*, 2010; Rossello *et al.*, 2014; Wang *et al.*, 2013b; Haiko *et al.*, 2016). Recently, other applications have been described, focusing on the detection of antibiotic resistance mechanisms (Jung *et al*., 2014; Hrabák *et al.*, 2014; Oviaño *et al.*, 2014; Wang *et al.*, 2013a).

The first group to use MALDI-TOF directly on urine was Ferreira *et al*. (2010). They analyzed 260 urine specimens detected as potentially infected by a flow cytometry screening device (UF-1000i, bioMérieux). The samples were processed in parallel by culture. Correct identifications were obtained, at species and genus levels, for 79.2% and 80% of isolates, respectively by MALDI-TOF. The hugely dominant specimen was *E. coli*- accounting for 94.2% of all isolates (Ferreira *et al.*, 2010).

In our study the agreement between MALDI-TOF and conventional culture was slightly lower, at 71.8%. This may partly be because 44% of the positive urines had high (>10^5) numbers of WBC/mL, which may make analysis more difficult. Another key differences from Ferreira is that *E. coli* was only present in 25 out of the 96 culture-positive urines. This low proportion of *E. coli* reflected a deliberate strategy of sample collection, seeking to represent pathogen diversity rather than the dominance of *E. coli* presented in the epidemiological study. Importantly, we represented Gram-negative as well as Gram-positive bacteria, and these are harder to lyse owing to a more robust cell wall, thus presently a greater challenge.

MALDI-TOF identified different species for 12/81 urines compared with culture performed at the clinical laboratory, however the same isolates from direct identification were re-identified on MALDI-TOF from these cultivated urines indicating that these urines may contain mix population of bacteria with overgrowth of the second species between initial culture and sub-culture during mass spectrometry analysis, or that original identification were in error.

The sample preparation method directly from urine for MALDI-TOF was originally described by Ferreira *et al.* (Ferreira *et al.*, 2010) and has been adopted or slightly modified by others including (Wang *et al.*, 2013b; Rossello *et al.*, 2014; Hong *et al.*, 2014) as well as in this study. This procedure consists of a slow centrifugation step to pellet human cells (2,000 g for 30 sec) then a faster centrifugation step to collect the bacteria (15,500 g for 5 min), followed by washing and protein extraction. Sánchez-

Juanes *et al.* (2014) incorporated the step of processing the urine samples using 10% SDS (Sánchez-Juanes *et al.*, 2014). In our study, and in order to remove the highest number of human cells, we found it useful to increase the duration of the first centrifugation to 2 min at 2,000 rpm (300*g*) and then treated the bacterial pellet with 1% SDS and lysozyme, which improved detection and identification for Gram-positive bacteria. We found that these modification led to much higher MALDI-TOF scores, and greater accuracy in the identification of Gram-negative and Gram-positive pathogens from urines with high numbers of human cells.

The major limitations of MALDI-TOF for urine analysis nevertheless is the diagnostic threshold of 10^5 cfu/mL and the challenge of identification in mixed cultures. Direct MALDI-TOF failed for 15/96 infected urines mainly due to counts below the detection limit (<10^5 cfu/mL). In the study by (Schubert *et al.*, 2011), where analysis was performed on growth from blood culture bottles, at least one isolate was correctly identified in mixed cultures; in our study both bacterial species were correctly identified in 16/27 mixed urines.

In general better scores for identification were achieved for Gram-negative bacilli than Gram-positive cocci. This difference may reflect the thicker peptidoglycan cell wall of Gram-positive bacteria (De Carolis *et al.*, 2014). The thinner cell walls of Gram-negative bacilli are more easily lysed and intracellular proteins more easily released for detection.

A potential advantage of MALDI-TOF over conventional urine microbiology is that it can detect urogenital pathogens not able to grow on standard media for urinary infections e.g. *Acinobaculum schalii* or *Gardnerella vaginalis*; both these species were encountered in the present study.

Identification by MALDI-TOF is more precise than with chromogenic agars which do not distinguish between e.g. *Klebsiella* spp., *Enterobacter* spp. or *Citrobacter* spp. which are “lumped” as ‘coliforms’, nor among species from the Proteeae family. This is important as e.g. *Klebsiella* spp. are usually sensitive to co-amoxiclav whereas *Enterobacter* spp. or *Serratia* spp. are resistant. Another example is that *Proteus mirabilis* generally is susceptible to co-amoxiclav and gentamicin whilst *Providencia* spp. and *Morganella morganii* mostly are resistant. Many laboratories in the UK do not identify uropathogens to species level and so cannot readily draw this infections

on susceptibility (Chin *et al.*, 2016). A recent survey of laboratory methods in England carried out in 63 laboratories showed that 80% used chromogenic agar to identify *E. coli*, and that only 15% used MALDI-TOF (on cultures, not directly) for further differentiation (A. MacGowan, unpublished results).

Although the current sample preparation is not automated the present study shows that MADLI-TOF could be used to deliver the organism's identity within < 1 hour in urgent cases e.g. urosepsis. If combined with resistance genes identification using PCR, or detection of antibiotic resistance mechanisms also on MALDI-TOF, it could significantly increase the patient's likelihood of receiving early targeted appropriate therapy.

4.3 Detection of β-lactamase activity directly from urine samples and their cultivated isolates from MALDI-TOF

Among Enterobacteriaceae, most resistance to β-lactam antibiotics is conferred by production of β-lactamase enzymes. AmpC, ESBLs and carbapenemases all play important roles in the resistance to modern β-lactams. Recently MALDI-TOF has been shown to be able to differentiate β-lactamases within 1-4 hours from cultivated isolates of various species (Jung *et al*., 2014; Jung *et al.*, 2016; Sparbier *et al.*, 2012; Lasserre *et al.*, 2015), also directly from urines (Oviaño *et al.*, 2017) and from the early growth harvested from blood cultures (Oviaño *et al.*, 2014; Ghebremedhin *et al.*, 2016; Carvalhaes *et al.*, 2014). These approaches are based on the monitoring of hydrolysis of the central β-lactam ring of the antibiotic during incubation with the bacteria. Disappearance of the mass peak for the native β-lactam and possible appearance of a new peak corresponding to the hydrolysed form indicates hydrolysis and predicts resistance.

Most studies have focused on the detection of carbapenemases (Oviaño *et al.*, 2017; Hoyos-Mallecot *et al.*, 2014b; Sakarikou *et al.*, 2017) because (i) of the high profile of these enzymes, which undermine the utility of "last resort" carbapenem antibiotics against multi-drug resistant Gram-negative bacteria, and (ii) of the worldwide spread of carbapenem-resistant Enterobacteriaceae (Logan & Weinstein, 2017; Kim *et al.*, 2017; Findlay *et al.*, 2017). However for practical purposes it might

be better to seek ESBLs, as these are commoner than carbapenemases in UTIs and have more straightforward treatment implications (avoid cephalosporins, and owing to frequently linked resistance-fluoroquinolones). Detection of ESBLs has a great potential impact on the antimicrobial therapy because cephalosporins are safe and effective agents to treat UTIs so long as these enzymes are absent. Swift identification of patients appropriate for cephalosporin therapy might reduce carbapenem use. We therefore evaluated the utility of MALDI-TOF to detect cephalosporin hydrolysis in infected urines and in the isolates cultivated from them. For this purpose we tested ESBL-and AmpC-producing Enterobacteriaceae resistant to 3rd and 4th generation cephalosporins. These included cefotaxime, ceftazidime and cefpodoxime, which are widely recommended for phenotypic ESBL screening, also cefepime, which is more stable to AmpC enzymes, and ceftriaxone.

4.3.1 Peak pattern for native and hydrolysed cephalosporins

The first study aiming to detect hydrolysis of different β-lactam antibiotics using MALDI-TOF was reported by (Sparbier *et al.*, 2012). They tested clinical isolates of *E. coli* producing ESBLs and carbapenemase-producing and non-producing *K. pneumoniae* strains with sodium/or potassium salts of ampicillin, piperacillin, ceftazidime, cefotaxime, ertapenem, meropenem, and imipenem. They then analysed the peak profile of the different degradation products and showed that each antibiotic gave its own unique peak profile. Hydrolysis resulted in the disappearance of the native peak pattern of all tested antibiotics. These data were supported by our study, where each of the cephalosporins tested had a different peak profile, which was altered by incubation with the β-lactamase positive (CTX-M-15 or TEM-10) controls but not by the β-lactamase-negative controls.

The applicability of the MALDI-TOF-based hydrolysis assays using cefotaxime and ceftazidime has been previously described by (Sparbier *et al.*, 2012; Oviaño *et al.*, 2014; Li *et al.*, 2014), but no data were generated for cefepime, cefpodoxime and ceftriaxone.

Analysis of native cephalosporins and these incubated with susceptible bacteria revealed the mass spectrum peaks at 481 Da for cefepime, 428.58 Da for

cefpodoxime, 554.58 Da for ceftriaxone, 456 Da for cefotaxime and 547 Da for ceftazidime, all of which were lost during incubation with resistant isolates. Direct hydrolysis products (with a mass shift +18 Da) or decarboxilated hydrolysed products (mass shift of -44 Da) were not observed for any cephalosporin and it is likely that complex fragmentations occur.

We also detected the cephalosporin group structure peaks at 396-398 Da for cefepime and ceftriaxone and two peaks at 577.15 Da and 621.73 Da for ceftriaxone only. Analysis of native cefotaxime and ceftazidime showed also peaks at 478 Da for cefotaxime and 468 Da for ceftazidime-possibly reflecting elimination of pyridine ring. All of these peaks were lost during incubation with the positive controls without appearance of simple hydrolysis products, again suggesting fragmentations.

4.3.2 Detection of cephalosporin-hydrolysing activity

Although EUCAST and CLSI guidelines now recommend reporting susceptibility testing results based on phenotypic behaviour only without editing of categorical results (EUCAST, 2017; CLSI, 2013) distinguishing cephalosporin-resistant strains with AmpC and ESBL enzymes is important epidemiologically and to select adequate antibiotic therapy. In reviewing the cephalosporin-hydrolysis results in relation to phenotypic susceptibility data and genotypes, we observed good agreement for ESBL producers but less so for AmpC hyperproducing strains. Cephalosporin-susceptible isolates almost always gave no hydrolysis of any cephalosporins.

Almost all ESBL producers hydrolysed 4 or 5 of the cephalosporins tested. On this basis, a reasonable method to swiftly detection of ESBL producing Enterobacteriaceae would be to set up a panel of 3rd and 4th generation cephalosporins, and if hydrolysis of any four or more cephalosporins is detected then to rule out the use of all cephalosporins for treatment. Even if EUCAST and CLSI recommendations to report resistances as 'found' for ESBL producers are accepted, it should be stressed that most ESBL producers are resistant to cephalosporins.

Jung *et al.* (2014) previously demonstrated 100% sensitivity in the detection of class A β-lactamase producers among Enterobacteriaceae using cefotaxime from

positive blood culture (Jung *et al.*, 2014). Another study that showed good accuracy in distinguishing cefotaxime-resistant and cefotaxime-susceptible *E. coli* strains, also from positive blood cultures, was presented by (Foschi *et al.*, 2016). Although the authors did not characterize the cefotaxime-resistance mechanism the sensitivity of the assay was 94.7%. Oviaño *et al.* (2014) analysed hydrolysis products based on the disappearance of native mass peaks of cefotaxime and ceftazidime alone and in combination with clavulanic acid. The study achieved 99% sensitivity for the detection of ESBL producing bacteria from positive blood cultures (Oviaño *et al.*, 2014). Here we achieved sensitivity for ESBL detection at 91.4% directly from urines, and 98.1% for the isolates grown from them.

The assay was much less reliable for detecting AmpC activity in urines or their isolates. This no doubt reflects the slow hydrolytic activity of AmpC enzymes for oxyimino-cephalosporins e.g. cefotaxime, ceftazidime and ceftriaxone (low V_{max} value) although their affinity is high (low K_m values) (Jacoby, 2009; Livermore, 1987). Only 1/22 urine containing AmpC producers gave detectable full hydrolysis for two cephalosporins and 6/22 urines containing AmpC producers gave hydrolysis for one cephalosporin, for the remaining 15/22 urines slow hydrolysis or no hydrolysis were seen for the cephalosporins tested. Whilst using the corresponding isolates, hydrolysis was detected for at least one cephalosporin with 18/22 organisms, though only 1/22 gave demonstrable full hydrolysis all 5 cephalosporins. Overall the sensitivity of the assay for AmpC producers was low- 32.4% directly from urines and 69.7% for bacterial isolates. Another study also demonstrated lower detection of sensitivity for AmpC producers than ESBL producers (83% *vs.* 99%) from positive blood culture; similarly as in our study no hydrolysis was observed for ceftazidime and cefotaxime for strains carrying bla_{CMY-2} (Oviaño *et al.*, 2014).

The total hands-on time directly from infected urines to a hydrolysis profile was 2.5 h, including sample preparation and MALDI-TOF analysis for most cephalosporins except ceftazidime when a longer incubation was needed (4.5 h). All cephalosporins could be analysed on the same MALDI-TOF target plate, together with direct pathogen identification from the urine specimen, giving a total turnaround time <5 h. The assay could measure 1-6 samples/target plate with no additional cost.

Although the initial costs of mass spectrometry purchase (and the service cost) are relatively high, the cost per sample of bacterial identification and hydrolysis testing remain low compared with molecular techniques. A similar algorithm has already been applied for carbapenemases by (Oviaño *et al.*, 2016; Oviaño *et al.*, 2017). These authors used MALDI-TOF for identification of Gram-negative bacilli directly from urine using a commercial Sepsityper kit (Brüker) to detect carbapenem-hydrolysing isolates using imipenem as a substrate. The assay showed 100% sensitivity and specificity for detecting carbapenemase activity including OXA-48 producers within 90 min from sample reception.

Rapid detection of ESBL producers from urine by MALDI-TOF might be used to guide therapy especially in urgent cases. For example temocillin or carbapenems might be preferred over cephalosporins, co-amoxiclav or ciprofloxacin, to which most ESBLs producers are resistant. Although this approach could accelerate adaptation of definitive therapy there are five major limitations that have to be overcome:

Firstly, unreliable detection of hydrolysis of AmpC producers. Hyperproduction of AmpC can arise as a result of mutational derepression of chromosomal enzymes in e.g. *Enterobacter* spp. (Sanders & Sanders, 1992; Livermore, 2008) or when AmpC types become plasmid-encoded and are acquired (Philippon *et al.*, 2002; Hanson, 2003). The former is commoner and the risk is predictable from species identification or local epidemiology (with a high risk for *Enterobacter* spp.). To detect cases it might also be useful to explore adding first-generation cephalosporins (e.g. cefazolin and cephalothin) that are good substrates for AmpC β-lactamases (Jacoby, 2009) to the test panel.

Secondly, the methods cannot detect secondary resistance mechanism, such as alterations in outer membranes or efflux pumps which together with the presence of ESBL and AmpC enzymes can engender resistance to ertapenem (Livermore *et al.*, 2012). More generally, the MALDI-TOF hydrolysis assay needs to be further validated for "impermeable" *E. coli.*

Thirdly, a high input volume (1.5 mL urine/antibiotic) is required, with a bacterial density $>10^5$ cfu/mL.

Fourthly, the manual sample preparation procedure may be too laborious for clinical laboratories. It might be possible to minimize hands-on time by developing a simple kit, as proposed for MALDI-TOF-based carbapenemase detection (Oviaño *et al.*, 2016)

Lastly, the automated analysis software should be standardized with an option to review peak patterns in order to minimize the risk of analytical discrepancies. In our study we analyzed the data using commercial automated MBT-STAR-BL software for ceftazidime and cefotaxime and the MBT-STAR-BL prototype for the remaining cephalosporins. Additionally, we compared the raw peak profile of hydrolysed and non-hydrolysed cehpalosporins using manual FlexControl software. We noticed discrepancies between automated software and manual analysis for 5/91 urines and 7/91 cultivated isolates. To resolve these we found it useful to review both raw peak profiles and logRQ values obtained from automated analysis. In one study ClinPro Tools software (Li *et al.*, 2014) was used to review changes in a mass spectra and establish a mathematical model to distinguish between ESBL-producing and nonproducing bacteria automatically, avoiding the problem of standardization of assay evaluation (Li *et al.*, 2014), and this may provide an alternative route to resolution of the issue.

Overall, the hydrolysis assay is a promising approach for rapid detection of ESBL producers among Enterobacteriaceae directly in urine samples. Together with direct pathogen identification, and possibly PCR to seek other antibiotic resistances, it could facilitate the implementation of targeted therapy for urosepsis patients.

4.4 Multiplex PCR for detection of bacterial resistance genes

Although MALDI-TOF can detect β-lactamase activity it remains a challenge to detect other resistance mechanisms, and particularly those where an antibiotic is not degraded or clinically modified by bacterial enzymes e.g. changes in DNA gyrase, porins or efflux pumps. Several attempts have been made to establish detection tests for methicillin-resistant *Staphylococcus aureus* (Edwards-Jones *et al.*, 2000), vancomycin-resistant enterococci (Griffin *et al.*, 2012) and resistance against aminoglycosides by measuring the acetyltransferase activity of *aac(6')-Ib-cr* in

Enterobacteriaceae (Burckhardt, 2013; Oviaño *et al.*, 2017; Pardo *et al.*, 2016) using MALDI-TOF. However, the reproducibility of these approaches is insufficient for routine use in a clinical laboratory.

PCR-based methods may be better, thorough they are specific for the particular genes sought. They have been employed to seek antibiotic resistances to a variety of Gram-positive (Wang *et al.*, 2016; Chung *et al.*, 2016; Okolie *et al.*, 2015) and Gram-negative bacteria (Moran *et al.*, 2017; Singh *et al.*, 2016; Lau *et al.*, 2015; Ogutu *et al.*, 2015). Chavada and Maley (2015) previously evaluated the utility of multiplex tandem PCR, as used here, for detecting 12 different β-lactamase genes. These included plasmid AmpC, ESBLs and carbapenemases in Gram-negative strains (Chavada & Maley, 2015).The authors argued that, with some improvement, the MT-PCR assay could be used in clinical laboratories.

We sought to use multiplex, tandem PCR for resistance genes profiling directly from urine specimens and cultivated isolates. For this purpose we evaluated three multiplex tandem PCR assays seeking common resistance genes among Enterobacteriaceae. The first assay sought 8 resistance genes including four internationally widespread trimethoprim determinants (*dfrA1, dfrA5/14, dfrA7/A17* and *dfrA12*), two aminoglycoside genes (*aadA1/A2/A3* and *aac(6')-Ib*) and also *gyr* and *parC*, where mutations can confer resistance against fluoroquinolone. The rationale was that fluoroquinolone (e.g. ciprofloxacin) and trimethoprim are widely used for treatment UTIs. The second assay was expanded to seek also β-lactamase genes including classical penicillinases (bla_{TEM}, bla_{SHV}), β-lactamase inhibitor combination ($bla_{\text{OXA-1}}$), AmpC (bla_{CMY}), ESBLs ($bla_{\text{CTX-M gp 1}}$, $bla_{\text{CTX-M gp 9}}$) and carbapenemases ($bla_{\text{OXA-48}}$, bla_{KPC}, bla_{NDM}, bla_{VIM}). Lupo *et al.* (2013) showed that these are the commonest β-lactamases in the Enterobacteriaceae worldwide, and these genes should be targeted for the development of non-phenotypic tests for a rapid detection of β-lactam resistance antibiotics (Lupo *et al.*, 2013). Besides these β-lactamase targets the 16-Plex MT-PCR assay then aimed to (a) detect a broader range of resistance genes common in Gram-negative bacteria than the 8-Plex assay plus (b) to distinguish 'Enterobacteriaceae' to genus level by different melting temperatures for an unspecified gene amplification product. The third 24-Plex assay was expanded to seek further genes for aminoglycoside-modifying enzymes including *aadB, aacC1,*

aacC2 and *aacC3.* These all confer gentamicin and/or tobramycin resistance. In total, the assay aimed to detect the commonest antibiotic resistance genes in Enterobacteriaceae including four targets to identify non-Enterobacteriaceae to group level (pan-Enterococcus, pan-Streptococcus, pan-Staphylococcus, pan-Pseudomonas).

4.4.1 Trimethoprim resistance determinants

Trimethoprim affects bacterial folic acid synthesis, by inhibitioning the enzyme dihydrofolate reductase (DHFR) (Huovinen, 2001). There are several trimethoprim resistance mechanism including mutations in the promoter region or in the dihydrofolate reductase gene (*dfr* itself), but the most common mechanism is acquisition of the trimethoprim-resistant *dfr* gene through mobile genetic elements including plasmids and transposons, which led to rapid spread of trimethoprim resistance among Enterobacteriaceae. At present there are more than 30 different *dfr* genes (White & Rawlinson, 2001; Seputiené *et al.*, 2010) associated with class 1 and 2 integrons harbouring *dfr* gene cassettes.

There have been a few studies of the distributions of underlying trimethoprim resistance genes in Europe and other continents (Blahna *et al.*, 2006; Grape *et al.*, 2007a; Seputiené *et al.*, 2010; Lee *et al.*, 2001; Yu *et al.*, 2004). The results of these confirm that *dfrA1, dfrA5, dfrA7, dfrA12, dfr14* and *dfrA17* are the commonest trimethoprim determinants in general accounted for 75-86% of trimethoprim resistance (Grape *et al.*, 2007a). All were included in our MT-PCR assays.

Most epidemiology surveillance showed that *dfr1* and *dfrA17* are the commonest acquired *dfr* genes found in uropathogenic *E. coli* (Blahna *et al.*, 2006; Lee *et al.*, 2001, Yu *et al.*, 2004; Brolund *et al.*, 2010; Towner *et al.*, 1994). The view was supported by our study, where *dfrA7/A17* predominated among all trimethoprim-resistant *E. coli* specimens, accounted of 33% in the 8-Plex and 24-Plex and 43% in the 16-Plex assays. There is a paucity of published data regarding the prevalence of *dfr* genes in *K. pneumoniae*. Surprisingly in the 16-Plex assay, we observed that *dfrA5/A14* dominated in *K. pneumoniae*, similar as in one study (Brolund *et al.*, 2010) performed

in Sweden. The lowest prevalence was observed for *dfrA12* and *dfrA1* determinans in all three assays.

None of the *dfr* alleles sought were found in 5/73 samples with trimethoprim-resistance in 16-Plex assay. This may indicate the presence of other *dfr* determinants undetected in this panel. Seputiené *et al.*, (2010) showed that *dfrA8* was as common as *dfrA5* isolated from urine in Lithuania. In the report from South Africa again *dfrA8* was frequent isolated among the Gram-negative commensal faecal flora (Adrian *et al.*, 1995). In Brazil, *dfrA25* was isolated from urine sample, from *Salmonella agona* (Agersø *et al.*, 2006). In other studies *dfrB2*, *dfr24* and *dfrA26* were found in *E. coli* isolates (Grape *et al.*, 2007b; Dworniczek, 2007) while the *dfr9* allele was found in veterinary isolates in Sweden and has also been isolated from patients with UTIs (Jansson *et al.*, 1992).

No *dfr* genes were found in 5/21 urines and/or isolates containing bacteria resistant to trimethoprim in the 24-Plex assay. Nonetheless in 4 out of these samples, a weak signal was observed, suggesting that the gene was present but weakly amplified. In the remaining one sample, 24-Plex assay did not found any of the sought *dfr* genes indicating that other *dfr* alleles may be present, similar as in the 16-Plex assay.

The overall performance for detection of *dfr* genes, conferring trimethoprim resistance, was more reliable in the 8-Plex and 16-Plex assyas than the 24-Plex, demonstrating good agreement vs. phenotypic testing in both urines and isolates; as noted ealier we believe that technical improvement of the 24-Plex assay is needed.

4.4.2 Aminoglycoside resistance determinants

The spread of plasmid-encoded aminoglycoside modifying enzymes and 16S rRNA methyltransferases (ArmA, Rmt and NpmA) are the main factors contributing to increase resistance to aminoglycosides (Ma *et al.*, 2009; Jana & Deb, 2006). The commonest genes for aminoglycoside-modifying enzymes in *E. coli* are *aac(6')-Ib*, targeted in all three assays, *aac(3)-II* (alternative name *aacC2*) and *ant(2")-I* (*aadB*) both included in the 24-Plex, *aac(6')-I* (*aacA1*), *ant(3")-I* (*aadA*) and *aph(3')-II* (*aphA*-

2) (Xiao & Hu, 2012; Shaw *et al.*, 1993). Based on PHE records (data not published) we also included *aac(3)-I* and *aac(3)-III*, which are frequent isolated in the UK, in the 24-Plex assay.

All MT-PCR assays sought *aac(6')-Ib* which is associated with tobramycin resistance, and its *cr* variant conferring low-level resistance to ciprofloxacin (Robicsek *et al*.,2006; Strahilevitz, *et al.,* 2009), though there were not distunguish. In principle such discrimination might be achieved on the MT-PCR system. Guillard *et al.* (2013) combined SybrGreen real-time PCR with pyrosequening to detect *aac(6')-Ib* and *aac(6')-Ib-cr* by their different melting temperatures. Nevertheless, the results showed melting peaks very close to each other at 87.89 °C and at 87.71 °C, which may be difficult to resolve on the real-time instrument with lower sensitivity (Guillard *et al.*, 2013). In reality though, these aspects are of little impact as most samples containing *E. coli* with detected *aac(6')-Ib* anyway correspond to *A1/R* gyrase type, predicting mutational resistance to fluoroquinolones.

The detection of *aac(6')-Ib* was reliable in 8- and 16-Plex assays, but lower in the in 24-Plex where 6/20 samples with tobramycin resistance profile gave weak signals, below the threshold value applied (concentration >550 copies of molecules, $C_T \leq 21$), suggesting that the gene was present but probably poor amplified. Similar problems were seen for *aac(3)* variants, though it is also possible that issue here reflected diversity within the *aac(3)-I* family, where types b and e were not included in the 24-plex assay were also seen but they were identified by sequencing.

In one previous study *aac(6')-Ib-cr* was included in a multiplex PCR panel seeking eight plasmid-mediated quinolone-resistance determinants in the UK (Ciesielczuk *et al.,* 2013). The authors showed that high prevalence of *aac(6')-Ib-cr* in *E. coli* strains, isolated from patients with bacteraemia and UTIs was associated with ESBLs mostly CTX-M-15, as did others (Deepak *et al.*, 2009; Amin & Wareham, 2009) . This probably reflects *E. coli* ST131 accounting for most *E. coli* in the UK and being resistant to all these agents. Although the 8-Plex assay did not seek β-lactamase genes this linkage may be useful to rule out also cephalosporins (for *aac(6')-Ib*-positive results) in the treatment of UTIs if clinical laboratories that decide to apply 8-Plex assay in routine practice.

Hu *et al.* (2013) developed a GeXP analyzer-based multiplex PCR to detect seven aminoglycoside-resistance genes including five aminoglycoside modifying enzymes genes (*aac(3)-II*, *aac(6')-Ib*, *aac(6')-II*, *ant(3")-I* and *aph(3')-VI*) and two 16S rRNA methyltransferase genes (*armA* and *rmtB*) (Hu *et al.*, 2013). The authors showed that *ant(3")-I, aac(3)-II* and *armA* predominated in Enterobacteriaceae clinical isolates in China while in our study *aac(6')-Ib* and *aac(3)-II* were the most prevalent types though the latter was sought only in the 24-Plex. Similar results were also found in studies in Norway (Lindemann *et al.*, 2012; Haldorsen *et al.*, 2014), whilist Ho *et al*. (2010) demonstrated that 84.1% of gentamicin-resistant *E. coli* isolated from patients with UTIs in Hong Kong were positive for *aac(3)-II* (Ho *et al.*, 2010). An other study showed that mutations in *aac(6')-Ib* affects the activity towards amikacin (Shmara *et al.*, 2001). None of the present isolates with *aac(6')-Ib* in all tested assays expressed phenotypic resistance to amikacin. Nonetheless the use of amikacin in this case for the UTIs treatment is controversial where AAC(6') is present as the drug is a substrate for inactivation. EUCAST recommends to report amikacin as intermediate in Enterobacteriaceae if the strains is tobramycin- intermediate or resistant and gentamicin susceptible.

Poor predictive power was found for *aadA1/A2/A3* tests in all three MT-PCR assays. These *aadA1/A2/A3* was detected in both streptomycin-resistant and -susceptible bacteria impying (i) the presence of other determinants (e.g. *strA/strB* and *aadA5*), not sought in these assays (Sundin, 2002) and (ii) the presence of unexpressed *aadA* genes. Batchelor *et al.* (2008) found *aadA1* and *aadA4* in several streptomycin-susceptible *E. coli* isolates suggesting that the gene was not expressed (Batchelor *et al.*, 2008). Other example of potential gene silencing was demonstrated in (Enne *et al.*, 2006). Sunde & Norström, (2005) demonstrated that *strA/strB* genes are involved in high-level streptomycin resistance whereas *aadA* gene more often confers low-level streptomycin resistance. In practical terms this matters as there are no CLSI and EUCAST-defined streptomycin breakpoints, although resistance is often demarcated by an MIC of ≥64 mg/L (Dudley *et al.*, 2013) but (Tyson *et al.*, 2015) showed that the value often not reacted by isolates carrying *aadA* genes. Additionaly, streptomycin is not ordinarily used in UTIs. On these bases the simplest answer would

be to remover *aadA* target from the assay. These would have little or no clinical impact and would remove an unreliable panel.

4.4.3 β- *Lactamase resistance genes*

Although selective agars can be used to seek ESBL producers and those with other key β-lactamase types, these require overnight incubation and has lower sensitivity than PCR-based methods (Naas *et al.*, 2011; Singh *et al.*, 2012). The use of multiplex PCR or real-time PCR (commercial or in-house) for the detection of β-lactamase genes in Enterobacteriaceae has been evaluated in many studies (Singh *et al.*, 2016; Poirel *et al.*, 2011; Ogutu *et al.*, 2015; Nijhuis *et al.*, 2012; Lau *et al.*, 2015), but none of these assay was applied directly to clinical urines as here.

Singh *et al.* (2016) developed a multiplex real-time PCR assay for the detection of 10 β-lactamase genes including ESBL, AmpC and carbapenemase using, as here, melting curve analysis (Singh *et al.*, 2016). The diversity of allelic variants for all sought β-lactam genes were much higher than in our study. However the common $bla_{\text{CTX-M-9}}$ group genes ($bla_{\text{CTX-M-9}}$, $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-16}}$, $bla_{\text{CTX-M-24}}$) were not included in the panel while all of these genes, excluding $bla_{\text{CTX-M-16}}$ were sought in our MT-PCR β-lactamase tests in the 16-and 24-Plex assays. Findlay *et al.* (2015) compared three commercial kits (Check-Direct CPE kit (Check-Points, Netherlands), the Eazyplex® SuperBug CPE kit (Amplex, Germany), the Xpert® Carba-R kit (Cepheid, CA)) for carbapenemase detection (see Section 1.8.8.1). The authors concluded that all these kits can reliable detect clinically significant carbapenemases (Findlay *et al.*, 2015). Similarly, here the β-lactamase panel in the 16- and -24-Plex assays gave reliable results in detecting carbapenemase genes directly in clinical urines and cultured isolates.

The accuracy of detection of β-lactamase genes by the 16- and 24-Plex assays directly from urine was comparable with the studies by Chavada and Maley (2015) and Willemsen *et al.* (2014). In the former study the authors used the same technology as here- multiplex tandem PCR-to detect 12 β-lactamase genes including ESBLs, AmpC and carbapenemases in cultivated Gram-negative isolates, achieving

sensitivity of 95% and specificity of 96.7% (Chavada & Maley, 2015). Willemsen *et al.* (2014) evaluated commercial real-time PCR (Check-MDR ESBL PCR) for detection three ESBL β-lactamase targets (*bla*$_{\text{CTX-M-like}}$, *bla*$_{\text{TEM-ESBL like}}$, *bla*$_{\text{SHV-ESBL}}$) where ESBL and non-ESBL variants of *bla*$_{\text{TEM}}$ and *bla*$_{\text{SHV}}$ were distinguishable. The study showed 98.9% sensitivity and 100% specificity against a reference microarray assay (Willemsen *et al.*, 2014). In all these cases pure cultures were used, not clinical urines as here. Although our panel could not differentiate ESBL and non-ESBL variants of *bla*$_{\text{TEM}}$ and *bla*$_{\text{SHV,}}$ both assays showed 100% sensitivity for clinical urines and cultivated isolates. The specificity was higher for the 16-Plex than 24-Plex (95.3% *vs.* 60% for clinical urines) underscoring technical issues with the latter.

4.4.4 Quinolone resistance genes

High-level quinolone resistance in Enterobacteriaceae occurs mainly due to mutations within the Quinolone Resistance-Determinaning Regions (QRDR) of *gyrA* or *parC* (Shigemura *et al.*, 2012). The commonest mutations are Ser83 and Asp87 in *gyrA* and Ser80 and Glu84 in *parC* (Nakano *et al.*, 2013; Bansal & Tandon, 2011; Friedman *et al.*, 2001; Park *et al.*, 2017). Low-level resistance or reduced susceptibility to quiolone is associated with plasmid mediated quinolone-resistance (PMQR) mechanisms e.g. Qnr proteins, *aac(6')-Ib-cr* (Robicsek *et al.*, 2006; Jacoby *et al.*, 2009); mutations of genes (*QepA, OqxAB*) regulating the expression of efflux pumps and alterations that decrease the permeability of the bacterial cell wall (Périchon *et al.*, 2007; Strahilevitz *et al.*, 2009), Efflux pump up-regulates are more important in *P. aeruginosa* than in Enterobacteriaceae.

Here, MT-PCR predicted the ciprofloxacin-resistance based on *gyrA* melting temperature product. Because it is commercial kit the specific mutations sought in quinolone genes are unknown. *gyrA/R* was found in most *E. coli* tested with ciprofloxacin-resistance in all three assays, whereas quinolone resistance in other species could not be predicted from the test results.

Nakano *et al.* (2013) previously developed a rapid assay to detect *gyrA* and *parC* mutations by PCR-RFLP in *E. coli, K. pneumoniae*, *E. cloacae* and *Salmonella* spp.

within 3 h (Nakano *et al.*, 2013), however the use of this method in clinical practice is impractical due to possible sample contamination during the sample processing and low throughput. In an other study multiplex allele specific PCR was used to detect 'hot spot' mutations in fluoroquinolone-resistant *E. coli* isolates. Depending on the mutations sought the sensitivity of the method was in a range 93.33%-100%, and specificity 98.48%-100% (Onseedaeng & Ratthawongjirakul, 2016). In our study the sensitivity was in the range 83.3%-100% and specificity 100% for the detection of *gyrA* mutations for in *E. coli*. The major limitation of the panel is inability to distinguish the fluoroqionolone-resistance other than in *E. coli*, thus limit treatment guidance for UTIs caused by other coliform species.

4.4.5 Limitation of the study

In conclusion, all three AusDiagnostics assays rapidly identified resistance genes in infected urines, cultivated isolates, or with bacterial DNA. Use with urines could potentially guide early therapy. Potential advantages of these assays are (i) being fully automated, (ii) working direct on urine, without culture and DNA extraction, (iii) a rapid turnaround time (2-3h), (iv) low reagent cost (£12-15 sample/run), (v) easy-to-handle interpretation software. The 8-Plex assay was set up in a format of a 72-tube ring to test 9 samples and the 16-Plex as a 384-well plate to run 24 samples. Although both these latter assays allowed more specimens to be run, the reagents and consumables could not be separated for individual (or smaller number of) samples, whereas the 24-plex assay is set up in a format of 3x8-well strip, allowing 1-4 samples to analysed per run.

A major limitation in our study was the number of specimens tested in the 24-Plex assay (n=23), and the limited sequencing undertaken on these samples to confirm MT-PCR results. This assay also needs to be review because it gave several weak results (e.g. with *aac(6')-Ib* or *aac(3')*) probably related to manufacture.

Several further limitations remain to be solved.

Firstly, the inability to detect rarer resistance determinants e.g. to trimethoprim or novel, uncharacterized mechanisms of resistance, for which the genetic determinant is unknown. The absence of the limited number of common targets sought cannot exclude the possible presence of other genes conferring resistance to relevant antibiotics. For example, in the 16- and 24-Plex assays six samples contained bacteria resistant to trimethoprim, but no sought trimethoprim determinants were found using either assays, suggesting the presence of rarer *dfr* genes, as disscussed above (Section 4.4.1). The other observed example is *aadA1/A2/A3,* conferring resistance to streptomycin. Here the detection of the resistance gene did not always correlate with phenotypic resistance. A susceptible profile, despite detection of *aadA,* might be associated with poor expression of the gene, silencing or inactivation. On the other hand negative results by MT-PCR and a resistant phenotype probably indicates the presence of other genes associated with streptomycin resistance e.g. *strA/strB*.

Secondly, phenotypic resistance to cephalosporins and carbapenems among Enterobacteriaceae may depend not only on the presence of the β-lactam genes, but on their level of expression plus decreased outer membrane permeability and/or increased efflux (Woodford *et al.*, 2007) or mutations effecting the expression of chromosomal AmpC (Livermore, 2008). The development of techniques that rely on the measurement of gene transcripts (mRNA levels) instead of the presence of a gene might provide a potential solutions to these problem.

Thirdly, detection of fluoroqionolone resistance mutations was possible only in *E. coli*, and the inability to distinguish ESBL and non-ESBL variants of bla_{TEM} and bla_{SHV} precludes advice on cephalosporin use in treatment where there are found, though it should be added that TEM and SHV ESBLs are rare in *E. coli* with CTX-M types grossly predominated (Ryoo *et al.*, 2005; Calbo *et al.*, 2006)

Fourthly, as with all molecular methods, multiplex-tandem real-time PCR does not provide MIC values, which are the basis of pharmacodynamically guiding antimicrobial therapy.

Fifthly, although the 24-Plex assay contained targets to identify *Pseudomonas* spp., *Enterococcus* spp., *Streptococcus* spp., and *Staphylococcus* spp. no resistance

genes for Gram-positive bacteria were included in the assay and most resistance in *P. aeruginosa* is mutational, not via gene acquisition, making it difficult to detect by PCR. Deletion of the identification targets and extending the list of resistance determinants for Gram-negative bacteria might be more useful, for example by addition of *gyr* tests for distinguishing ciprofloxacin-resistant and -susceptible coliforms other than *E. coli,* detection of *ant(4')-I, ant(4')-II* and *aph(3')-VI* genes conferring amikacin resistance, detection of 16S rRNA methyltranserases ArmA and RmtB/C, which confer high-level resistance to all standard aminoglycosides (Hidalgo *et al.*, 2013) or to detect clonal groups among *E. coli* ST69, ST73, ST12, ST95 and especially ST131, which is much more often multi-drug resistant than the other STs . Such a panel could allow not only identication of resistance genotypes but could also be useful for epidemiology and infection control purposes.

Sixthly, the cut-off criteria need to be optimized in order to minimalize the risk of false positive/negative results. In our study the analysis was performed on the basis of (i) a high product concentration (>1000 copies in the 16-Plex assay and >550 in the 24-Plex assay, both in the 2nd PCR step), (ii) a cycle curve (C_T) < 20 and < 21 in the 2nd PCR step for the 16- and 24-Plex assays respectively, (iii) a correct melting temperature for the amplified genes (T_m). These cut-off value precluded several 'false positive results'; however we probably missed several weak positive results for *aac(6')-Ib, aac3'-I* and *dfrA5/A14* potentially in the 24-Plex assay. Weak positives may be associated with (i) poor gene amplification or (ii) low bacterial counts in urines tested.

Lastly, the early version of interpretation software with an option to guide antibiotic choices for targeted therapy needs to be improved by developing appropriate algorithms to rule out antibiotic if a gene is present. Several times the software called that patient may respond to gentamicin even though aminoglycoside determinants conferring gentamicin resistance were present.

In summery, the designs of the 8-,16-,24-Plex assays were based on the local and UK epidemiology and antibiotic resistances. The resistance targets were limited to the commonest resistance determinants in Enterobacteriaceae. Although, there are some challenges to overcome we remain optimistic that these assays will be widely

useful in clinical laboratories. Together with MALDI-TOF identification assay they could accelerate the initiation of definitive therapy for urosepsis patients. Using a combinated approches give a total turnaround time from urine submission to the clinical laboratory to pathogen and resistance gene identification of *c.* <3 h, compared with 24-48 h by standard phenotypic methods.

4.5 Nanopore sequencing- a step forward in pathogen identification and antibiotic resistance genes profiling

Whilst MALDI-TOF technology, together with multiplex PCR, could provide fast and complex diagnostics for urosepsis patients, fundamental issues remain. PCR can seek only a limited number of targets and thereby can predict a resistance, but cannot exclude it. In principle, new sequencing approaches, such as MinION could deliver rapid and much more comprehensive diagnostics for high-risk urosepsis patients.

MinION nanopore technology was released in 2014. Since then, the developer (ONT) has claimed major improvements in miniaturization and automation of the equipment, also modernization of applications and software to facilitate results analysis when the system is employed in microbiology practice. Nevertheless, the use of this device directly on clinical samples was not previously described. Our study showed that it gives the possibility not only to identify the organism, but also to detect the key resistance genes, directly in urine in a time frame similar (or even faster) to the combined MALDI-TOF/PCR approach (i.e. <5 hours).

We tested either (i) clinical urines with a high number of bacteria *vs.* human cells and limited commensal flora or (ii) urines spiked with a multi-drug resistant *E. coli* isolate. Hasman *et al.* (2014) previously applied Ion Torrent sequencing to urine, finding identical resistance genes as in the cultivated pathogens but, with a 24-h turnaround (Hasman *et al.*, 2014), their method only modestly accelerated conventional workflows. The utility of metagenomic sequencing, using the Illumina platform, directly on clinical samples was also explored in other studies (Brown *et al.*, 2015; Christiansen *et al.*, 2014); nonetheless sequencing for clinical purposes must

be both rapid and accurate if the results are lead to improved individual patient care or public health investigation and Illumina based sequencing cannot meet these criteria whereas Nanopore potentially do so. Preliminary experiments showed that the MinION technology could be useful in diagnostic microbiology to identify microorganisms, predict resistances in cultivated bacteria (Kilianski *et al.*, 2015; Wang *et al.*, 2014b; Bradley *et al.*, 2015; Quick *et al.*, 2016; Judge *et al.*, 2015) and to track microorganism outbreaks (Quick *et al.*, 2015; Gardy *et al.*, 2015). Recently Votintseva *et al* (2017) demonstrated the utility of MinION sequencing in rapid (<8 h) identification of *M. bovis* BCG strain and susceptibility using 'sputum spiked' with the cultured strain (Votintseva *et al.*, 2017).

The potential advantages of the MinION over other sequencing platforms are presented in Section 1.8.8.3. The technology remained active under development whilst these studies were undertaken and continues to be improved.

The manufacturer's improvements in quality of the flow cells, together with refinements in our sample preparation, led to significant increases in sequence yields and 2-D sequencing reads through the course of these studies. Briefly, experiments with Clinical Urines 1-4 failed even to identify the pathogen, while later experiments sucesfully identified both the species and its resistance genes directly from urine. The first attempts of using MinION sequencing on isolated microorganisms by other research groups were also unsuccessful. Mikheyev and Tin (2014) reported high error rates, with only 10% reads from 36h of sequencing of lambda phage mapping to the reference genome (Mikheyev & Tin, 2014) while Quick *et al.* (2014) concluded that bioinformatics streaming algorithms for individual analysis needed to be developed (Quick *et al.*, 2014). In our study preliminary experiment with CU1, without human cell depletion, showed a large proportion of human reads, and correspondingly low bacterial sequence yield. Subsequently we enriched bacterial DNA, initially by NEBNext® Microbiome DNA Enrichment kit (CUs 2-4), which still gave unsatisfactory results due to the low concentration of bacterial DNA obtained. From CU5, we adopted a combination of differential centrifugations to remove most human cells, together with MolYsis to lyse the remaining human cells and to achieve maximum recovery of bacterial DNA. This allowed us to identify pathogens with high accuracy

to reference genomes (>90%), and to detect the same families of acquired resistance genes as found by Illumina, with good agreement to resistance phenotypes.

Several systems incorporating real-time analysis of MinION data have been developed recently (Quick *et al.*, 2015; Greninger *et al.*, 2015). In our study most sequence analysis was post-run, using BLAST and CARD databases applied via manual bioinformatics pipelines. However, in the final part of these studies using the latest version of flow cell (SQK-MAP-006) with Spiked Urine run 3, we applied Metrichor WIMP and ARMA softwares in order to automate and accelerate analysis. This allowed us to identify the pathogen in 15 min and profile its acquired resistance genes in 1h, during which time *c.* 32 MB of 2-D sequencing data were generated, with almost 7x depth of coverage (57 MB of 2-D data with 11.37x depth were available after 2h). Cao *et al.* (2016) utilized a different framework for analysis of MinION data; their pipeline included various streaming algorithms for pathogen identification which could be achieved within 30 min of sequencing using only 500 reads with initial resistance gene detection within 2h (Cao *et al.*, 2016). Bradley *et a.l* (2015) used a de-Bruijn graph approach to identify antibiotic resistance genes for *Staphylococcus aureus* and *Mycobacterium tuberculosis* from 8h of MinION sequencing run (Bradley *et al.*, 2015). Neither of these approaches gave advantages of speed over the WIMP/ARMA approach, through both were swifter than the manual BLAST/CARD strategy.

To predict the likehood of detecting *all* acquired antibiotic resistance genes, the size of the bacterial genome needs to be considered. Based on Lander and Waterman's (Lander & Waterman, 1988) equation, we calculated that statistically 6.96x depth should cover 99.905% of the *E. coli* genome (4.6 MB), leaving little risk of missing an acquired resistance gene. This prediction extends to *K. pneumoniae* and *Enterobacter cloacae* as they have a similar genome size to *E. coli* (5.3 MB for *K. pneumoniae* and 4.8 MB for *Enterobacter cloacae*). In the case of *P. aeruginosa*, which has larger genome (6.3 MB), 6.96x depth would cover only 99.873% marginally increasing the risk of missing some acquired genes.

MinION sequencing error rates were an early concern, but are diminishing as the technology has been refined with >95% base calling accuracy now achievable (Szalay

& Golovchenko, 2015). Recent R9.4 pore chemistry also has improved the read accuracy and coverage depth (Jansen *et al.*, 2017); yields might also be further increased to 230Mb/hour (Miten *et al.*, 2017). On the bases of these improvements we suggest that MinION sequencing of clinical urines is already potentially more efficient and effective than achieved here on the technology available in 2014-16.

MinION sequencing, as done here, including DNA extraction and sample transport (2.5h), library preparation (3h), and sequencing (1h) combined with the simultaneous data analysis (i.e. WIMP/ARMA) approximated to a 7-8 h turnaround, equating to one dosage interval for a 'typical' q8h antibiotic. Further improvement, with the use of the 15-min library preparation kit, as with Spiked urine run 4 potentially reduced this turnaround to *c.* 4-5h (though WIMP/ARMA could not be used with the 1-D reads generated with this kit). Further development of the automated sample processor VolTRAX (ONT) will allow acceleration of the DNA extraction and library preparation, standardization of the procedure and thereby minimizing the risk of human mistakes. Such modification will facilitate implementation of MinION technology in clinical settings, allowing ealier refinement of antibiotic therapy than now.

Although this approach has great potential compared with other sequencing platforms and molecular methods there are several limitations which have to be overcome.

Firstly, in our study we used urine with "significant" bacteriuria (>10^8 cfu/L or >10^5 cfu/mL) to deliver the 1 µg of input DNA required for library preparation. Complicated UTIs can occur with much lower bacterial counts (10^{2-} 10^4 cfu/mL) though counts are usually high in serious urosepsis. The use of the Rapid Low-Input Kit (ONT) reducing the DNA requirement from 1 µg to 10 ng (ONT) for library preparation may address this issue, though this was not tested.

Secondly, the MinION technology is adjusted to test only one urine (specimen) per flow cell. Although, this is convenient and flexible, allowing sequencing straightaway when samples arrive to the laboratory, it is expensive (£400-720/flow cell with R7.3 chemistry plus *c*. £120 reagent cost/sample). However, taking into

account the facts (i) we might save a day's hospitalization (which costs £400) (Department of Health, 2015) or (ii) avoid using expensive antibiotic therapy (e.g. ceftazidime-avibactam £275 per day) (Mosley *et al.*, 2016), individual testing might be an appropriate solution for some high-risk patients. Alternatively, Oxford Nanopore have introduced four solutions: (i) the GridION X5, as a compact benchtop system design to analyse and run up to five MinION flow cells individually or concurrently, (ii) the PromethION, a high-throughput sequencing platform that allows docking of 48 flow cells, (iii) a PCR-free rapid barcoding sequencing kit to pool and run up to 12 samples on each flow cell, and (iv) the MinION MkI model based on 'pay-as-you-go', allowing 3h of sequencing with an estimated cost of £210, generating 3 GB of data in 'fast mode' software, while sequencing by the MinION MkII model sequencing will cost £17/hour, generating 5 GB of data (ONT, 2015). Such approaches could greatly reduce the cost per sample but would necessitate sample batching, extending the wait for results. The MALDI-TOF/PCR approach would continue to have a significantly lower cost and assuming resolution of the 24-Plex assays', greater flexibility.

Additionally, Oxford nanopore have developed a new smaller-than-current SmidgION sequencing device which would allow data analysis even via a mobile phones. The principle is the same as MinION but greater portability will allow sequencing e.g. on the ward rather than only in the laboratory.

Thirdly, at present identification is limited to acquired resistance genes, mostly carried by mobile genetic elements (or transferred to the chromosome from these) as also found by (Judge *et al.*, 2015). It remains a challenge to identify mutational changes conferring resistance via alterations in permeability, efflux pump expression target sensitivity. Thus, chromosomal *gyrA* and *parC* genes were found in all bacterial sequences from clinical urines but the read accuracy of MinION was insufficient for reliable calling of mutations generating high-level fluoroquinolone resistance, whereas these could be identified by Illumina sequencing. Likewise, allelic variants within β-lactamase families (e.g. TEM and SHV) were poorly distinguished and MinION failed to detect mutations associated with *ampC* up-regulation, and could not discriminate acquired and plasmid-borne *ampC*. In this regard plasmid-encoded

AmpC enzymes almost always are copiously expressed and have clear resistance association, whereas the implications of chromosomal AmpC depends on level of expression. In *E. coli* this is determined by promoter and attenuator mechanisms (Honoré *et al.*, 1986) whereas in species with inducible chromosomal AmpC e.g. *E. cloacae* or *C. freundii*, expression depends on mutations in regulatory genes (principally *ampR* and *ampD*). The plasmid-encoded types are derived from the chromosomal-encoded *ampC* genes of other Enterobacteriaceae. As an example, CMY-2 is originally from *Citrobacter freundii* whilst MIR-1 is from *Enterobacter cloacae* (Bauernfeind *et al.*, 1996). Plasmid types are liable to be miscalled when the species with the corresponding chromosomal enzyme is tested.

MinION frequently flagged multiple alleles (e.g. of bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$, bla_{NDM}) when Illumina indicated single types. Differenting bla_{NDM} or $bla_{CTX\text{-}M}$ variants is unimportant, as all their alleles have similar resistance implications. However, SNPs in bla_{TEM}, bla_{SHV}, bla_{GES} determine hydrolytic spectrum and inhibitor vulnerability, thereby indicating whether a therapy is appropriate or not. Currently, distinguishing these closely related β-lactamase variants, and predicting AmpC expression, is challenging for MinION technology owing to low read accuracy. Although SNPs can be called using MinION data (Quick *et al.*, 2016) the process is slow. Improvements also are needed in the bioinformatics pipeline and automated software to better discriminate among related resistance genes and polymorphisms or to indicate what is effectively duplicate calling of a single, impartially identified gene. In the future this may be achieved by using better chemistry (R9) that minimize the error rate and simultaneously generates yields with high accuracy reads that allow creating single consensus sequence. Alternatively, and more laboriously reads aligning to CARD or other antibiotic resistance genes databases could be isolated and polished to improve consensus accuracy, facilitating precise identification.

Fourthly, as in all molecular methods based on resistance gene detection, the presence of a gene may not correspond with phenotypic resistance due to poor expression, silencing or inactivation. For example MinION and Illumina found *aadA5* in CU6 but the *E. coli* isolate was streptomycin susceptible. Similar contradictions, particularly with *aad* genes were frequent observed in MT-PCR assays (above).

Likewise Tyson *et al.* (2015) previously noted poorer genotype-phenotype concordance for streptomycin that for other resistances (81.3% *vs.* 100%) (Tyson *et al.*, 2015).

Fifthly, optimising the MinION gene-match cut-off to only call 'true positive' results for resistance genes is challenging. We used 90% for Illumina and (owing to lower base-calling accuracy), 80% for MinION. This lower cut-off may explain the larger number of misdetections of plasmid *ampC* and aminoglycoside determinants *aadA3* and *aacA4* by MinION in clinical urines, and the calling of *catB3* gene in *E. coli* H141480453 by MinION but not Illumina. A technical aspect, independent of MinION, was occasional misdetection of resistance genes due to inclusion of flanking regions of integrons in CARD database (not shown). This could potentially lead to an over-estimation of the occurrence of resistance genes in patient samples. Here it was countered by checking the reads mapped within genes but would better be resolved by stricter database curation.

Lastly, implementation of WIMP and ARMA software definitely accelerated the data analysis, but for practical utilization, software is required to convert the genotypic profile into clinically relevant prediction of phenotypes to guide treatment option.

All the clinical urines tested in this study were infected with single pathogens. Polymicrobial UTIs were not sought, though WIMP software can identify and differentiate multiple species in one sample (Juul *et al.,* 2015). Distinguishing multiple strains with the same species is unlikely to be achieved, but all their resistance genes would be represented in the sequence data.

The utility of metagenomic sequencing using isolates has been demonstrated in a diagnostic and public health microbiology (Köser *et al.*, 2012; Harris *et al.*, 2013; Köser *et al.*, 2013), though a recent EUCAST subcommittee report took the view that WGS with inferred antibiotic susceptibility testing is insufficient to guide clinical decision making (Ellington *et al.*, 2017). Given the improvements achieved already in MinION technology and those likely in the near future we are optimistic that the technology can be enhanced to overcome the residual challenges. If so, MinION

profiling from urosepsis patients could allow beneficial refinement of antibiotic regimens within the first dosage interval after clinical diagnosis. It will also support molecular epidemiology surveillance and infection control to monitor the antibiotic resistance.

Chapter 5

CONCLUSION AND FUTURE DIRECTIONS

The UK 5-year Antimicrobial Resistance Strategy emphasized the role of point-of-care diagnostics to identify where antimicrobials are required and to assess the appropriateness of the diagnosis and treatment (PHE, 2013).

The present study aimed to evaluate new technologies for rapid detection of pathogens and their antibiotic resistances in urosepsis patients. For this purpose two potential approaches: (i) MALDI-TOF together with multiplex tandem PCR (or MALDI-TOF based ESBL detection) and (ii) MinION nanopore sequencing, were investigated, optimized and applied. Neither technique has previously been used directly on urine samples in healthcare settings, except for limited investigation of direct pathogen identification from urine by MALDI-TOF.

Both approaches proved to be able to deliver rapid pathogen and resistance profiling before the second dose of typical (i.e. every 8 h) antibiotic ordinarily would be given. Both methods have limitations and several improvements could be introduced. However, we believe that either could, in principle, be implemented into the diagnostic pathway in urgent urosepsis cases. Either system could be used to complement subsequent phenotypic testing, giving a swifter rule-out of antibiotics to which the bacteria were predicted to be resistant.

Epidemiological surveillance here and elsewhere points the settings where these approaches might be justified, including (i) high-risk patients showing clinical signs and symptoms of urosepsis, particularly elderly (>65y) with complicated UTIs, with indwelling devices, and likely to have high rates of bacterial resistance owing to previous exposure to antibiotics or transfer from other countries/hospitals where resistance rates are high, and (ii) hospital units (EAU, CCC, ICU) where resistance rates are generally high, or where outbreaks are ongoing.

By MALDI-TOF we demonstrated that (i) identification of uropathogen directly from urine is achievable, (ii) Enterobacteriaceae producing ESBLs can be detected by

MALDI-TOF based cephalosporin hydrolysis assays again directly from urine, and (iii) both these assays can be performed in parallel with a turnaround time of <5h. This approach might be especially useful for patients admitted to the hospital due to cUTIs.

As an alternative to hydrolysis assays, three multiplex-tandem PCR were designed to seek common and important resistance genes in Enterobacteriaceae directly from urine samples. Each of the assays showed relatively good agreement between reference phenotypic and molecular methods. Implementation of the appropriate software to guide treatment options could facilitate interpretation and indicate clinically relevant information, especially for patients with a high risk of carrying resistant bacteria. The timeframe of the MT-PCR is <3 h.

Combinations of the MALDI-TOF/MT-PCR methods could improve and accelerate current diagnostics for urosepsis patients. Both these assays are (i) easy-to-perform, use equipment that is familiar in the clinical laboratory (ii) fast, with overalll turnaround times <3 h, (iii) work directly on urine so as to minimize the delay caused by DNA extraction and culture, and (iv) are relatively cheap in terms of reagents and consumables purchases (£0.5-1.00/sample for MALDI TOF plus £12-15/sample for MT-PCR). Nevertheless these approaches require further validation and optimization in a routine laboratory setting, moreover, resistance investigation is not comprehensive.

In the case of MinION, we demonstrated that the technology can offer a rapid and potentially comprehensive approach to identify pathogens and acquired resistances in urine samples, without culture. Although there are several limitations and challenges to overcome e.g. the cost of the reagents/flow cells, low throughput, it is the first sequencing-based technology that could be potentially implemented into clinical settings in the possibly near future. Along with cost reduction, improvements are needed in read accuracy and interpretative software (ARMA), particularly to better discriminate among related resistance genes and to reliably call single nucleotide polymorphisms. Development of analysis software that translate resistance genes into phenotypic profile is required in order to introduce nanopore sequencing into healthcare system.

Decisions whether, where and how to implement either of these two approaches in diagnostic practice requires understanding and knowledge of the whole process used in the current clinical settings along with precise cost modelling. Two scenarios are proposed for (i) the secondary care (hospitals) and (ii) the primary care (GPs and private clinics).

5.1 Implementation of the rapid diagnostics in secondary care

The models examined during this thesis are more appropriate to secondary care e.g. hospitals. Among the two evaluated approaches (MALDI-TOF/MT-PCR or MinION), MALDI-TOF/MT-PCR would be easier to adopt into hospital workflows because (i) it is less laborious, (ii) reagents and consumables costs are cheaper, and (iii) MALDI-TOF is already available and used for pathogen identification from the cultivated isolates, whilst multiplex PCR systems are increasingly used in virology and for carbapenemase detection from rectal swabs (to inform infection control). Nevertheless the more comprehensive analysis achieved using the MinION sequencing-based approach could represent the future of rapid diagnostics for urgent cases and might also be used for surveillance and outbreak investigation.

On the basis of my experience, a workable model would be that urine from suspected urosepsis patient needs to be flagged as for 'urgent attention' at the time of collection, then the ward should call to the clinical laboratory. Simultaneously the 'urgent workflow' should be stressed on the form with a sticker when the sample is submitted to the clinical laboratory. The sample could then be processed urgently using MALDI-TOF/MT-PCR or MinION approach. This workflow is similar to the process traditionally used for CSF specimens where meningitis is suspected. Because many centralized UK microbiology laboratories now work in shifts it should be possible to use these methods to deliver a results in a timeframe of <5h, with findings released to clinicians on the same day.

Key limitations that must be addressed before implementation in diagnostic workflow are: (i) instrumentation costs, (ii) staff pressure (lack of time for individual processing samples in understaffed departments), (iii) staff training to interpret

genotyping results, (iv) that the presence of a resistance determinant does not always correlate with phenotypic resistance, and (v) inability to identify novel or uncharacterized resistance mechanisms.

Automating data interpretation using computerized support systems (discussed below) need also to be implemented to aid antibiotic choice. These should minimize the risk of human mistakes and support stewardship. In the study performed by (Paul *et al.*, 2006) 'computerized TREAT model' was used to choice empirical treatment for UTIs. A randomized trial compared wards intervention using TREAT *vs*. antibiotic monitoring/prescribing without TREAT. This showed that the TREAT advice system prescribed appropriate empirical therapy significantly more frequently than physicians (70% versus 57%, $P < 0.001$) using fewer broad-spectrum antibiotics and halving physicians' antibiotic costs (Paul *et al.*, 2006). A good 'computerized support algorithm for empirical therapy should indicate the first-line treatment choice based on (i) patients results generated, (ii) local antimicrobial resistance rates, (iii) patients demography, (iv) history of previous medications and allergy, (v) underlying comorbidities and recent hospitalization and (vi) recent travels and migrations. Although our model supports targeted therapy, appropriate choice of empirical therapy would prevent treatment failure and resulting sides' effect.

5.2 Implementation of the point-of-care diagnostics in primary care

In primary care settings, e.g. GP surgeries, there is a need to apply for point-of-care tests that swiftly flag key resistances to antibiotics that ordinarily are used for treatment. Neither MALDI-TOF/PCR nor MinION is well adapted for use in a small, poorly-equipped medical centres. What rather would be useful is a simple system where urine is injected into e.g. a cartridge or strip to confirm or rule out infection and to identify trimethoprim resistance in around 15 minutes.

A new diagnostic introduced into GP workflow will need to fulfil basic expectations: (i) rapid turnarounds, (ii) being easy-to-use, (iii) having low cost and (iv) providing clinically relevant information.

By analogy a 'test and treat' service was implemented in some pharmacies in the UK and Republic of Ireland to seek streptococci group A in sore throat infections. The aim of the service, which utilizes an antigen detection test is to provide appropriate diagnosis and treatment, and thus to reduce unnecessary antibiotic usage in the case of viral infections. The results showed that two-thirds of examined patients did not need to visit their GP, whilst antibiotics were prescribed to under 10%, thereby saving the NHS 'millions' (Thornley *et al.*, 2016; Little *et al.*, 2013) (through the patients had to pay for the test at pharmacy).

A similar model could be used for uncomplicated UTIs (i.e. cystitis) in the community. Trained pharmacy or non-medical surgery staff could assess the patients presenting conditions using a clinical scoring algorithm. Patients with two or more positive symptoms of i.e. dysuria, frequent urination, abdominal pain, discomfort during urination and cloudy, foul-smelling urine, but without symptoms of upper UTIs (e.g. fever, blood in urine, flank or back pain) would be referred for point-of-care examination of urine. This would then include (i) a dipstick test to detect pathological changes and the presence of bacteria and WBC based upon nitrites and leucocytes analysis, and (ii) rapid PCR to seek *E. coli* and detect common trimethoprim resistance determinants in order to assess appropriate first-line treatment. If a *dfr* gene, indicating resistance to trimethoprim, is found nitrofurantoin would be prescribed whilst if a *dfr* gene is not detected, trimethoprim would be recommended. Patients with a clinical symptoms but negative dipstick result would be referred for GP consultation. A refinement would be to specifically seek ST131 *E. coli*, which accounts for much of the multi-resistance seen.

This scheme should avoid treatment failure, reduce inappropriate trimethoprim prescription and thus the spread of resistance to this agent. It would ensure that patients with bacteria susceptible to trimethoprim would still get this agent, which is superior to nitrofurantoin. Patient examination could take place in a private consultation room in a medical centre or pharmacy. The patient would pay for the test and antibiotics minimize the NHS costs, also ensuring that they were able to be seen immediately, rather than after the delay in getting a GP appointment.

This model would also need to ensure that patients with atypical or severe symptoms implying ascending infection i.e. fever, flank or back pain, shivering and chills, blood in urine, also patients from high-risk groups i.e. recurrent UTIs, children, pregnant women, diabetic, immunocompromised, cancer patients, travellers or imigrants from countries or regions with high resistance rates (e.g. South America, Asia, South Europe etc.) would be referral to visit their GP for further review. In this case, and based on the clinical details and medical history empirical treatment would be initiated simultaneously with urine sample collection, and sending to the clinical laboratory for culture or rapid PCR investigation e.g. for the combination of MALDI-TOF/PCR explored here.

5.3 Rapid point-of-care testing

To specifically seek *E. coli*, particularly ST131 and trimethoprim *dfr*-mediated resistance in urine specimens' in low technical settings such as GP surgeries, real-time LAMP (Loop-mediated isothermal AMPlification) detection system could be used. The technology is driven by the Amplex (Germany) and has proved to be effective and sensitive (Fernández-Soto *et al.*, 2014; Britton *et al.*, 2016). LAMP allows rapid cost-effective amplification of DNA at a constant temperatures thus eliminating the need for an expensive thermo cycler. In contrast to conventional PCR the methodology uses 4-6 different primers specifically designed to recognize 6-8 distinct regions on the target genes. Sample preparation takes 2-5 min, without DNA extraction, and analysis is performed in a small, portable, specifically designed for outdoor use Genie II or III (Amplex, Germany). The company has already developed ready-to-use rapid lyophilised kits (Easyplex) in a format of 8-well strip i.e. to identify the common pathogens causing cerebrospinal (CSF) infections, *C. difficille* toxins and for the detection of genes to identify VRE, MRSA and carbapenemases within 20-30 min. Development of a basic trimethoprim resistance test to seek common *dfr* trimethoprim determinants in *E. coli* within pharmacy (medical centre) consultation service thus seems feasible and would guide appropriate treatment. The total turnaround time should be no more than 1h.

Other example of rapid point-of-care tests would be GeneXpert II (Cepheid) and BioFire FilmArray (bioMerioux, France) both of which are based on the multiplex real-time PCR technology. In contrast to AusDiagnostic system the total turnaround time might be shorten to *c.* 1-2 h (MT-PCR 2-3 h), analysis is performed in one instrument (MT-PCR requires liquid-handling robot and real time platform) and the instruments are much smaller. The reaction is performed in specific cartridges to which the clinical sample is loaded which minimize the risk of reagents contamination or missing. Interpretation of results is performed by the internal software, indicating only clinical relevant information.

In principle a scheme involving point-of-care testing for rapid detection of *E. coli* and rule out of trimethoprim resistance in the community could (i) better guide treatment choice for UTIs, (ii) decrease the number of unnecessary urine submission by GPs to clinical laboratories for culture investigation, (iii) decrease the number of emergency admissions for treatment failures, thereby reducing cost of prolonged hospitalization, (iv) reduce the spread of resistance at the community through better targeted therapy.

5.4 Patients care *vs*. diagnostic cost

The cost of an overnight stay in an NHS hospital varies according to location and the type of services needed. Generally, the average hospital day costs £400 plus £500 for the operation and £250 for the consultation (Department of Health, 2015). The average total cost of a non-elective or elective inpatient, excluding excess bed days (which commonly arise owing to hospital acquired infections) is in a range £1,609-£3,749 for an Accident and Emergency admission (Department of Health, 2016). In 2013/14 the NHS spent £434 million for treating 184,000 hospital admissions for a urinary tract infection (The Medical Technology Group, 2015).

The cost of antibiotic therapy depends on the choice of treatment. Oral antibiotic therapy is much cheaper than intravenous. For example the cost per unit of oral ciprofloxacin (10 x 500 mg tablets) is £1.02 *vs.* £22.85 for intravenous ciprofloxacin IV (400 mg vial) (NICE, 2016).

The cost of antibiotics required to treat nosocomial complicated UTIs varies from £42 for gentamicin IV to £1,407 for ceftolozane/tazobactam 1g/0.5g IV based upon for 7-day course (NICE, 2016). To this must be added the cost of patient's investigation in term of equipment/reagents and staff costs.

The costs of new models must be seek to better manage UTIs in both community and hospitals. The costs for a community consultation service is likely to be more than for detection of group A streptococci where the patient pays £7.50 for the test and further £10 for antibiotics (Thornley *et al.*, 2016). Here, we proposed three steps approach which include (i) detection of bacteria using urine dipstick, (ii) identification of 'coliform' particularly *E. coli*, and (iii) detection of trimethoprim resistance; both the latter steps would be performed by rapid PCR method (e.g. LAMP technology). The initial costs for LAMP heating device or heat block may be high (e.g. Genie II costs £9,000 distributed by OptiGene, or Genie III costs around £14,000 distributed by Pro-Lab Diagnostics), however researchers who used this technology to detect Zika virus in human fluids (Chotiwan *et al.*, 2017) said that the device may costs around £195 (Colorado State University, 2017). The cost for consumables purchase for this technique may be similar to PCR (£10-20 sample/run, EasyPlex assay distributd by OptiGene) or even cheaper. Even if patient needs to pay £15-20 for the consultation service plus the cost of the antibiotic the benefits of the service are substantial. Only one hospitalization (for cUTI) per 100-200 patients must be allowed for the costs to roughly balance.

In the case of patients with complicated UTIs admitted urgently to hospital, or patients with hospital-acquired UTIs who are at high risk of developing urospesis, MALDI-TOF/PCR or MinION sequencing appear valuable. Although the initial costs for the equipment, reagents and annual service are relatively high (for example mass spectrometry costs £125,000 plus £13,000 annually for the service), the cost per sample for bacterial identification and hydrolysis testing is low (£0.5-1.00). The MT-PCR technique costs (£12-15 sample/run). The costs for the platforms purchase is around £40,000 (sample processor £14,280 plus Easy-Plex 96 £25,245, but analysis can be performed also using other real-time platforms e.g. LC480, Roche or Bio-rad). Nanopore sequencing currently is much more expensive (£520-840 sample/run) but

constant improvement of the technology should decrease this. Nonetheless the potential benefits are substantial and inclusive (i) reducing prolonged hospitalization and thereby diminishing healthcare costs (£400/day), (ii) prescribing appropriate (maybe cheaper) targeted antibiotics and minimizing the risk of treatment failure and its contingent costs (iii) decreased spread of multi-resistant organisms and of *Clostridium difficile* infections, with their long term costs and (iv) preserving limited treatment choices.

Implementation of the point-of-care tests in primary care and rapid diagnostics methods in secondary care should achieve a public health gain. Both patients with uncomplicated UTIs and complicated UTIs will be better cared for, with a more appropriate and effective alternative for management of UTIs.

The ultimate aim of these study was to improve both the clinical management of UTIs and antibiotic stewardship. If patients are treated more effectively at an early stage, this will diminish the number of complicated infections, bacteraemic episodes and the spreading resistances, along with their contingent personal, societal and financial costs. Moreover, where severe infection does arise, earlier information of the isolate's resistances will guide treatment choices, ensuring that an effective narrow-spectrum antibiotic is given. In the short term these techniques will not replace the standard culture, at least currently, but even now they could definitely accelerate the diagnostic workflow for urosepsis patients for substantial benefits.

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Appendix A

Statistics analysis of Epidemiological data

Table A 1. Testing the significance of the changes of urines submitted to the microbiology laboratory giving different types of results: July *vs*. November 2014.

Table A 2. Testing differences in the proportions of results for all urines submitted to the microbiology laboratory by Chi-square test, July *vs.* November 2014.

Table A 3. Testing the significance of the changes in the proportions of urines submitted from different locations, July *vs*. November 2014.

Table A 4. Testing the significance of the changes in the proportions of urines giving positive and negative results, July *vs*. November 2014.

Table A 5. Testing differences in the proportion of different bacterial species, July *vs*. November 2014 by Chi-square test.

Table A 6. Testing differences in the pathogen distribution by gender by Chi-square test, July *vs.* November, 2014.

Table A 7. Testing significance of changes in the proportions of isolates resistant to amoxicillin and trimethoprim by location, July and November 2014.

Table A 1. Testing the significance of the changes of urines submitted to the microbiology laboratory giving different types of results: July *vs.* November 2014.

Significance level	P < 0.05								
Results from the investigation	July p1_hat	November p2_hat	p_tilda	denom	z	critical1	critical2	p-value	
Iris screening negative	32.2%	31.6%	32%	0.006849455	0.881537787	-1.959963985	1.959963985	0.378026811	do not reject H0
Negative culture	29.4%	27.7%	29%	0.006640528	2.546986617	-1.959963985	1.959963985	0.01086576	reject H0
Positive culture	24.9%	25.6%	25%	0.006383389	-1.017958245	-1.959963985	1.959963985	0.3086978	do not reject H0
Heavy mixed bacterial growth	13.4%	15.0%	14%	0.005126557	-3.209437629	-1.959963985	1.959963985	0.001329949	reject H0

H0 (null hypothesis): both proportions are the same (i.e. nothing changed over time).

Table A 2. Testing differences in the proportions of results for all urines submitted to the microbiology laboratory by Chi-square test, July *vs.* November 2014.

Results from the investigation	July (n=9558)	November (n=8991)	Total (n=18549)	Predicted	Frequencies
Iris screening	3080	2843	5923	3052.026201	2870.973799
Negative culture	2814	2495	5309	2735.641921	2573.358079
Positive culture	2384	2301	4685	2414.104803	2270.895197
Heavy mixed bacterial growth	1280	1352	2632	1356.227074	1275.772926
p-value	0.002021461				
H0 (null hypothesis): independent; Reject H0; The distribution of results is not the same in July and in November.					

Table A 3.Testing the significance of the changes in the proportions of urines submitted from different locations, July *vs.* November 2014.

Significance level: 0.05									
Location	July	Nov	p_tilda	denom	z	critical1	critical2	p-value	
	p1_hat[1]	p2_hat[1]							
GP	67%	69%	68%	0.006865878	-3.368491117	-1.959963985	1.959963985	0.000755808	reject H0
H_IN	15%	13%	14%	0.005130618	2.869048685	-1.959963985	1.959963985	0.004117084	reject H0
H_OUT	6%	3%	4%	0.003025224	8.342225919	-1.959963985	1.959963985	0	reject H0
OH	2%	2%	2%	0.001944986	-1.282765742	-1.959963985	1.959963985	0.199574159	do not reject H0
AU	11%	13%	12%	0.004737059	-3.026030614	-1.959963985	1.959963985	0.002477872	reject H0

H0 (null hypothesis): both proportions are the same (i.e. nothing changed over time)

[1]Proportion of samples.

Table A 4. Testing the significance of the changes in the proportions of urines giving positive and negative results, July *vs*. November 2014.

Significance level	0.05							
Location/results	July	Nov	p_tilda	denom	z	critical1	critical2	
	p1_hat	p2_hat						
GP								
Iris screening	31.5%	31.8%	0.31617881	0.008294786	-0.321310552	-1.959963985	1.959963985	Do not reject H0
Negative culture	26.5%	24.9%	0.257556475	0.007800734	2.044108929	-1.959963985	1.959963985	Reject H0
Positive culture	28.3%	28.3%	0.283089405	0.008036405	-0.02227781	-1.959963985	1.959963985	Do not reject H0
Heavy mix bacterial growth	13.7%	15.0%	0.14317531	0.006248094	-2.096849652	-1.959963985	1.959963985	Reject H0
Hospital Inpatients	p1_hat	p2_hat	p_tilda	denom	z	critical1	critical2	
Iris screening	29.8%	24.3%	0.272658324	0.017403196	3.15185808	-1.959963985	1.959963985	Reject H0
Negative culture	39.2%	40.1%	0.396283656	0.019114808	-0.439176341	-1.959963985	1.959963985	Do not reject H0
Positive culture	17.4%	17.0%	0.172544558	0.014766356	0.287410601	-1.959963985	1.959963985	Do not reject H0
Heavy mix bacterial growth	13.5%	18.6%	0.158513462	0.01427273	-3.552343453	-1.959963985	1.959963985	Reject H0
Hospital Outpatients	p1_hat	p2_hat	p_tilda	denom	z	critical1	critical2	
Iris screening	29.0%	21.3%	0.263669502	0.032362575	2.392806162	-1.959963985	1.959963985	Reject H0
Negative culture	42.7%	38.3%	0.411907655	0.036149261	1.21740628	-1.959963985	1.959963985	Do not reject H0
Positive culture	17.7%	21.6%	0.190765492	0.028857841	-1.346701179	-1.959963985	1.959963985	Do not reject H0
Heavy mix bacterial growth	10.5%	18.8%	0.133657351	0.024992978	-3.304240875	-1.959963985	1.959963985	Reject H0

Location/results	July	Nov	p_tilda	denom	z	critical1	critical2	
	p1_hat	p2_hat						
Other Hospitals	p1_hat	p2_hat	p_tilda	denom	z	critical1	critical2	
Iris screening	22.6%	16.9%	0.196374622	0.043703952	1.322773801	-1.959963985	1.959963985	Do not reject H0
Negative culture	13.2%	14.5%	0.13897281	0.038056125	-0.348783949	-1.959963985	1.959963985	Do not reject H0
Positive culture	32.1%	39.5%	0.359516616	0.052791576	-1.412992861	-1.959963985	1.959963985	Do not reject H0
Heavy mix bacterial growth	32.1%	29.1%	0.305135952	0.050657975	0.593332891	-1.959963985	1.959963985	Do not reject H0
Admission Units	p1_hat	p2_hat	p_tilda	denom	z	critical1	critical2	
Iris screening	43.0%	43.6%	0.433211345	0.021206228	-0.27671866	-1.959963985	1.959963985	Do not reject H0
Negative culture	29.3%	29.3%	0.293229643	0.019482561	0.016599576	-1.959963985	1.959963985	Do not reject H0
Positive culture	17.5%	17.0%	0.17200366	0.016150501	0.303476878	-1.959963985	1.959963985	Do not reject H0
Heavy mix bacierial growth	10.2%	10.1%	0.101555352	0.012927068	0.04977565	-1.959963985	1.959963985	Do not reject H0

Table A 5. Testing differences in the proportion of different bacterial species, July *vs.* November 2014 by Chi-square test.

Pathogens	Observed			Predicted	
	July (n=2384)	November (n=2301)	Total	July (n=2384)	November (n=2301)
E. coli	1637	1565	3202	1629.735897	1572.264103
Other Coliform species	356	318	674	343.0487179	330.9512821
Enterococcus spp	137	107	244	124.1897436	119.8102564
Proteeae	98	119	217	110.4474359	106.5525641
Pseudomonas spp	76	105	181	92.12435897	88.87564103
CoNegStaph	26	21	47	23.92179487	23.07820513
Streptococcus Group B	24	25	49	24.93974359	24.06025641
S. aureus	17	15	32	16.28717949	15.71282051
S. saprophyticus	11	23	34	17.30512821	16.69487179
	2382	2298	4680		
p-value	0.02495943				
H0: independent; Reject H0; The distribution of the Gram-positive and Gram-negative isolates are not the same in July and in November ($p < 0.05$)					

Table A 6. Testing differences in the pathogen distribution by gender by Chi-square test, July *vs.* November, 2014.

Women	Observed			Predicted	
Pathogens	July (n=1793)	November (n=1769)	Total	July (n=1793)	November (n=1769)
E. coli	1352	1308	2660	1338.966292	1321.033708
Other Coliform species	237	227	464	233.5640449	230.4359551
Pseudomonas spp.	27	50	77	38.75955056	38.24044944
Proteeae	49	67	116	58.39101124	57.60898876
Enterococcus spp.	71	57	128	64.43146067	63.56853933
CoNegStaph	17	9	26	13.08764045	12.91235955
S. aureus	7	6	13	6.543820225	6.456179775
S. saprophyticus	11	23	34	17.11460674	16.88539326
Streptococcus Group B	21	21	42	21.14157303	20.85842697
	1792	1768	3560		
p-value	0.016251695				
H0: independent; Reject H0; The distributions of species/groups					

Men	Observed			Predicted	
Pathogens	July (n=591)	November (n=532)		July (n=591)	November (n=532)
E. coli	285	257	542	285.8526505	256.1473495
Other Coliform species	119	91	210	110.754717	99.24528302
Pseudomonas spp.	49	55	104	54.84995508	49.15004492
Proteeae	49	52	101	53.26774483	47.73225517
Enterococcus spp.	66	50	116	61.17879605	54.82120395
CoNegStaph	9	12	21	11.0754717	9.924528302
S. aureus	10	9	19	10.02066487	8.97933513
	587	526	1113		
p-value	0.547034194				
H0: dependent; DO not reject H0; The distributions of species/group					

Table A 7. Testing significance of changes in the proportions of isolates resistant to amoxicillin and trimethoprim by location, July and November 2014.

Significance level	5%							
	Amoxicillin, proportion resistant		p-tilda	denom	z	crit1	crit2	
Location	July	Nov						
GP	0.506319115	0.489911219	0.498203593	0.019981062	0.821172411	-1.959963985	1.959963985	Do not reject H0
H_IN	0.54491018	0.612903226	0.573883162	0.058621038	-1.15987448	-1.959963985	1.959963985	Do not reject H0
H_OUT	0.564516129	0.333333333	0.475247525	0.102063326	2.265091723	-1.959963985	1.959963985	Reject H0
AU	0.457627119	0.52892562	0.493723849	0.064684623	-1.10224808	-1.959963985	1.959963985	Do not reject H0
OH	0.458333333	0.571428571	0.53030303	0.127706392	-0.88558792	-1.959963985	1.959963985	Do not reject H0
	Trimethoprim, proportion resistant		p-tilda	denom	z	crit1	crit2	
Location	July	Nov						
GP	0.327804107	0.338175948	0.332934132	0.018832799	-0.550732832	-1.959963985	1.959963985	Do not reject H0
H_IN	0.449101796	0.346774194	0.405498282	0.058203417	1.758103011	-1.959963985	1.959963985	Do not reject H0
H_OUT	0.35483871	0.179487179	0.287128713	0.092464795	1.896413979	-1.959963985	1.959963985	Do not reject H0
AU	0.338983051	0.363636364	0.351464435	0.061769325	-0.399119028	-1.959963985	1.959963985	Do not reject H0
OH	0.25	0.404761905	0.348484848	0.121925884	-1.269311323	-1.959963985	1.959963985	Do not reject H0

Appendix B

Sensitivity and specificity for the cephalosporin hydrolysis assays by MALDI-TOF

Table B 1. Sensitivity and specificity for urines containing bacteria (A) and cultivated isolates from these urines (B) incubated with cefepime in buffer.

Table B 2. Sensitivity and specificity for urines containing bacteria (A) and cultivated isolates from these urines (B) incubated with ceftriaxone in buffer.

Table B 3. Sensitivity and specificity for urines containing bacteria (A) and cultivated isolates from these urines (B) incubated with cefpodoxime in buffer.

Table B 4. Sensitivity and specificity for urines containing bacteria (A) and cultivated isolates from these urines (B) incubated with ceftazidime in buffer.

Table B 5. Sensitivity and specificity for urines containing bacteria (A) and cultivated isolates from these urines (B) incubated with ceftriaxone in buffer.

Table B 6. Sensitivity and specificity for urines containing bacteria (A) and cultivated isolates from these urines (B) producing ESBLs for all tested cephalosporins.

Table B 7. Sensitivity and specificity for urines containing β-lactamases bacteria (A) and cultivated isolates from these urines (B) producing AmpC, for all tested cephalosporins.

Table B 1. Sensitivity and specificity for urines containing bacteria (A) and cultivated isolates from these urines (B) incubated with cefepime in buffer.

Sensitivity and specificity were calculated in two ways: (i) urines or cultivated isolates with intermediate zone were treated as susceptible and (ii) urines or cultivated isolates with intermediate zone were treated as resistant. Samples achieving 'slow hydrolysis' were treated as hydrolysed.

(A) Urines containing bacteria incubated with cefepime in buffer (Intermediate + Susceptible *vs.* Resistant)

Values entered:			
	Conditions		
	Cefepime S/I	**Cefepime R**	**Totals**
Cefepime hydrolysed	6	44	50
Cefepime not hydrolysed	35	4	39
Totals	41	48	89
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	0.539326	0.430829	0.644395
Sensitivity	0.916667	0.791284	0.972965
Specificity	0.853659	0.701378	0.939071
For any particular test results, the probability that it will be:			
Positive	0.561798	0.452767	0.665442
Negative	0.438202	0.334558	0.527233
For any particular positive test results, the probability that it is:			
True Positive	0.88	0.749973	0.950261
False Positive	0.12	0.049739	0.250027
For any particular negative test results, the probability that it is:			
True Negative	0.897436	0.748434	0.966626
False Negative	0.102564	0.033374	0.251566

(A) Urines containing bacteria incubated with cefepime in buffer
(Intermediate + Resistant *vs*. Susceptible)

Values entered:			
	Conditions		
	Cefepime S	**Cefepime R/I**	**Totals**
Cefepime hydrolysed	5	45	50
Cefepime not hydrolysed	35	4	39
Totals	40	49	89
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	0.550562	0.441773	0.654944
Sensitivity	0.918367	0.795162	0.973524
Specificity	0.875	0.723966	0.953052
For any particular test results, the probability that it will be:			
Positive	0.561798	0.3452767	0.665442
Negative	0.438202	0.334558	0.547233
For any particular positive test results, the probability that it is:			
True Positive	0.9	0.774088	0.962595
False Positive	0.1	0.37405	0.225912
For any particular negative test results, the probability that it is:			
True Negative	0.897436	0.748434	0.9626626
False Negative	0.102564	0.033374	0.251566

(B) Cultivated isolates incubated with cefepime in buffer
(Intermediate + Susceptible *vs.* Resistant)

Values entered:			
	Conditions		
	Cefepime S/I	**Cefepime R**	**Totals**
Cefepime hydrolysed	12	49	61
Cefepime not hydrolysed	27	1	28
Totals	39	50	89
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	0.561798	0.451767	0.665442
Sensitivity	0.98	0.879892	0.998955
Specificity	0.692308	0.522727	0.82451
For any particular test results, the probability that it will be:			
Positive	0.685393	0.577141	0.777431
Negative	0.314607	0.222569	0.422859
For any particular positive test results, the probability that it is:			
True Positive	0.803279	0.677811	0.889996
False Positive	0.196721	0.110004	0.312189
For any particular negative test results, the probability that it is:			
True Negative	0.964286	0.79761	0.998133
False Negative	0.035714	0.001867	0.20239

(B) Cultivated isolates incubated with cefepime in buffer (Intermediate + Resistant *vs.* Susceptible)

Values entered:			
	Conditions		
	Cefepime S	**Cefepime R/I**	**Totals**
Cefepime hydrolysed	11	50	61
Cefepime not hydrolysed	27	1	28
Totals	38	51	89
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	0.573034	0.463811	0.67589
Sensitivity	0.980392	0.882072	0.998976
Specificity	0.710526	0.538876	0. 84019
For any particular test results, the probability that it will be:			
Positive	0.685393	0.577141	0.777431
Negative	0.314607	0.222569	0.422859
For any particular positive test results, the probability that it is:			
True Positive	0.819672	0.696049	0.902358
False Positive	0.180328	0.097642	0.303951
For any particular negative test results, the probability that it is:			
True Negative	0.964286	0.79761	0.998133
False Negative	0.035714	0.001867	0.20239

Table B 2. Sensitivity and specificity for urines containing bacteria (A) and cultivated isolates from these urines (B) incubated with ceftriaxone in buffer.

Sensitivity and specificity were calculated in two ways: (i) urines or cultivated isolates with intermediate zone were treated as susceptible and (ii) urines or cultivated isolates with intermediate zone were treated as resistant.

(A) Urines containing bacteria incubated with ceftriaxone in buffer (Intermediate + Susceptible *vs.* Resistant)

Values entered:			
	Conditions		
	Ceftriaxone S/I	**Ceftriaxone R**	**Totals**
Ceftriaxone hydrolysed	0	41	41
Ceftriaxone not hydrolysed	35	12	47
Totals	35	53	88
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	0.602273	0.492138	0.703387
Sensitivity	0.773585	0.634502	0.87274
Specificity	1	0.876847	1
For any particular test results, the probability that it will be:			
Positive	0.465909	0.359943	0.574879
Negative	0.534091	0.425121	0.640057
For any particular positive test results, the probability that it is:			
True Positive	1	0.893306	1
False Positive	0	0	0.106694
For any particular negative test results, the probability that it is:			
True Negative	0.7244681	0.593626	0.855761
False Negative	0.255319	0.144239	0.406374

(A) Urines containing bacteria incubated with ceftriaxone in buffer (Intermediate + Resistant *vs*. Susceptible)

Values entered:			
	Conditions		
	Ceftriaxone S	**Ceftriaxone R/I**	**Totals**
Ceftriaxone hydrolysed	0	41	41
Ceftriaxone not hydrolysed	30	17	47
Totals	30	58	88
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	0.659091	0.549455	0.754672
Sensitivity	0.706897	0.570898	0.81537
Specificity	1	0.85868	1
For any particular test results, the probability that it will be:			
Positive	0.465909	0.359943	0.574879
Negative	0.534091	0.425121	0.640057
For any particular positive test results, the probability that it is:			
True Positive	1	0.893306	1
False Positive	0	0	0.106694
For any particular negative test results, the probability that it is:			
True Negative	0.638298	0.484781	0.769364
False Negative	0.361702	0.230636	0.515219

(B) Cultivated isolates incubated with ceftriaxone in buffer (Intermediate + Susceptible *vs*. Resistant)

Values entered:			
	Conditions		
	Ceftriaxone S/I	**Ceftriaxone R**	**Totals**
Ceftriaxone hydrolysed	2	48	50
Ceftriaxone not hydrolysed	35	6	41
Totals	37	54	91
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	0.593407	0.485227	0.693616
Sensitivity	0.888889	0.766852	0.954014
Specificity	0.945946	0.804695	0.990582
For any particular test results, the probability that it will be:			
Positive	0.549451	0.441922	0.652763
Negative	0.450549	0.347237	0.558078
For any particular positive test results, the probability that it is:			
True Positive	0.96	0.851412	0.993041
False Positive	0.04	0.006959	0.148588
For any particular negative test results, the probability that it is:			
True Negative	0.856659	0.701378	0.939071
False Negative	0.146341	0.060929	0.298622

(B) Cultivated isolates incubated with ceftriaxone in buffer (Intermediate + Resistant *vs*. Susceptible)

Values entered:			
	Conditions		
	Ceftriaxone S	**Ceftriaxone R/I**	**Totals**
Ceftriaxone hydrolysed	0	50	50
Ceftriaxone not hydrolysed	31	10	41
Totals	32	59	91
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	0.659341	0.551666	0.753395
Sensitivity	0.833333	0.710209	0.912985
Specificity	1	0.86273	1
For any particular test results, the probability that it will be:			
Positive	0.549451	0.441922	0.652763
Negative	0.450549	0.347237	0.558078
For any particular positive test results, the probability that it is:			
True Positive	1	0.911125	1
False Positive	0	0	0.088875
For any particular negative test results, the probability that it is:			
True Negative	0.756098	0.593558	0.870922
False Negative	0.243902	0.129078	0.406442

Table B 3. Sensitivity and specificity for urines containing bacteria (A) and cultivated isolates from these urines (B) incubated with cefpodoxime in buffer.

(A) Urines containing bacteria incubated with cefpodoxime in buffer.

Values entered:			
	Conditions		
	Cefpodoxime S	**Cefpodoxime R**	**Totals**
Cefpodoxime hydrolysed	0	54	54
Cefpodoxime not hydrolysed	25	10	35
Totals	25	64	89
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	0.719101	0.612257	0.806751
Sensitivity	0.84375	0.726762	0.918568
Specificity	1	0.834227	1
For any particular test results, the probability that it will be:			
Positive	0.606742	0.497247	0.706924
Negative	0.393258	0.293076	0.502753
For any particular positive test results, the probability that it is:			
True Positive	1	0.917265	1
False Positive	0	0	0.082735
For any particular negative test results, the probability that it is:			
True Negative	0.714286	0.534754	0.847631
False Negative	0.285714	0.152369	0.465346

(B) Cultivated isolates incubated with cefpodoxime in buffer.

Values entered:			
	Conditions		
	Cefpodoxime S	**Cefpodoxime R**	**Totals**
Cefpodoxime hydrolysed	1	62	63
Cefpodoxime not hydrolysed	24	3	27
Totals	25	65	88
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	0.722222	0.616196	0.808991
Sensitivity	0.953846	0.862413	0.988004
Specificity	0.96	0.77677	0.997908
For any particular test results, the probability that it will be:			
Positive	0.7	0.592946	0.789742
Negative	0.3	0.210258	0.407054
For any particular positive test results, the probability that it is:			
True Positive	0.984127	0.903164	0.999171
False Positive	0.015873	0.000829	0.096836
For any particular negative test results, the probability that it is:			
True Negative	0.888889	0.697034	0.970854
False Negative	0.111111	0.029144	0.302966

Table B 4. Sensitivity and specificity for urines containing bacteria (A) and cultivated isolates from these urines (B) incubated with ceftazidime in buffer.

(A) Urines containing bacteria incubated with ceftazidime in buffer

Values entered:			
	Conditions		
	Ceftazidime S	**Ceftazidime R**	**Totals**
Ceftazidime hydrolysed	0	29	29
Ceftazidime not hydrolysed	21	30	41
Totals	21	59	82
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	0.719512	0.607736	0.8110444
Sensitivity	0.491525	0.360532	0.623622
Specificity	0.913043	0.704913	0.984784
For any particular test results, the probability that it will be:			
Positive	0.378048	0.275277	0.492354
Negative	0.621951	0.507646	0.724723
For any particular positive test results, the probability that it is:			
True Positive	0.935484	0.77157	0.988746
False Positive	0.064516	0.011254	0.22843
For any particular negative test results, the probability that it is:			
True Negative	0.411765	0.278884	0.557871
False Negative	0.588235	0.442129	0.721116

(B) Cultivated isolates incubated with ceftazidime in buffer

Values entered:			
	Conditions		
	Ceftazidime S	**Ceftazidime R**	**Totals**
Ceftazidime hydrolysed	1	50	51
Ceftazidime not hydrolysed	29	11	40
Totals	30	61	91
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	0.67033	0.562922	0.763172
Sensitivity	0.819672	0.696049	0.902358
Specificity	0.966667	0.80947	0.998258
For any particular test results, the probability that it will be:			
Positive	0.56044	0.452676	0.663048
Negative	0.43956	0.336952	0.547324
For any particular positive test results, the probability that it is:			
True Positive	0.980392	0.882072	0.998976
False Positive	0.019608	0.001024	0.117928
For any particular negative test results, the probability that it is:			
True Negative	0.725	0.55862	0.848584
False Negative	0.275	0.151416	0.44138

Table B 5. Sensitivity and specificity for urines containing bacteria (A) and cultivated isolates from these urines (B) incubated with cefotaxime in buffer.

Sensitivity and specificity were calculated in two ways: (i) urines or cultivated isolates with intermediate zone were treated as susceptible and (ii) urines or cultivated isolates with intermediate zone were treated as resistant.

(A) Urines containing bacteria incubated with cefotaxime in buffer (Intermediate + Susceptible *vs*. Resistant)

Values entered:			
	Conditions		
	Cefotaxime S/I	**Cefotaxime R**	**Totals**
Cefotaxime hydrolysed	5	47	52
Cefotaxime not hydrolysed	25	12	37
Totals	30	59	89
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	0.662921	0.554034	0.757573
Sensitivity	0.79661	0.667968	0.886137
Specificity	0.833333	0.64549	0.936964
For any particular test results, the probability that it will be:			
Positive	0.58427	0.474905	0.686286
Negative	0.41573	0.313714	0.525095
For any particular positive test results, the probability that it is:			
True Positive	0.903846	0.782011	0.964056
False Positive	0.096154	0.035944	0.217989
For any particular negative test results, the probability that it is:			
True Negative	0.675676	0.501055	0.814449
False Negative	0.324324	0.185551	0.498945

(A) Urines containing bacteria incubated with cefotaxime in buffer (Intermediate + Resistant *vs*. Susceptible)

Values entered:			
	Conditions		
	Cefotaxime S	**Cefotaxime R/I**	**Totals**
Cefotaxime hydrolysed	3	49	52
Cefotaxime not hydrolysed	25	12	37
Totals	28	61	89
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	0.683393	0.577141	0.777431
Sensitivity	0.803279	0.677811	0.889996
Specificity	0.892857	0.7063	0.971912
For any particular test results, the probability that it will be:			
Positive	0.58427	0.474905	0.686286
Negative	0.41573	0.313714	0.525095
For any particular positive test results, the probability that it is:			
True Positive	0.942308	0.830784	0.984981
False Positive	0.057692	0.015011	0.169216
For any particular negative test results, the probability that it is:			
True Negative	0.675676	0.501055	0.814449
False Negative	0.324324	0.185551	0.498945

(B) Cultivated isolates incubated with cefotaxime in buffer (Intermediate + Susceptible *vs*. Resistant)

Values entered:			
	Conditions		
	Cefotaxime S/I	**Cefotaxime R**	**Totals**
Cefotaxime hydrolysed	3	49	52
Cefotaxime not hydrolysed	28	7	35
Totals	31	56	87
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	0.643678	0.533097	0.74143
Sensitivity	0.875	0.75314	0.944098
Specificity	0.903226	0.730997	0.974668
For any particular test results, the probability that it will be:			
Positive	0.597701	0.486925	0.699764
Negative	0.402299	0.300236	0.513075
For any particular positive test results, the probability that it is:			
True Positive	0.942308	0.830784	0.984981
False Positive	0.057692	0.015019	0.169216
For any particular negative test results, the probability that it is:			
True Negative	0.8	0.625358	0.909386
False Negative	0.2	0.090614	0.374642

(B) Cultivated isolates incubated with cefotaxime in buffer (Intermediate + Resistant *vs*. Susceptible)

Values entered:			
	Conditions		
	Cefotaxime S	**Cefotaxime R/I**	**Totals**
Cefotaxime hydrolysed	1	51	52
Cefotaxime not hydrolysed	28	7	35
Totals	29	58	87
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	0.666667	0.556528	0.761911
Sensitivity	0.87931	0.760922	0.946065
Specificity	0.965517	0.803719	0.998197
For any particular test results, the probability that it will be:			
Positive	0.597701	0.486925	0.699764
Negative	0.402299	0.30236	0.513075
For any particular positive test results, the probability that it is:			
True Positive	0.980769	0.884174	0.998995
False Positive	0.019231	0.001005	0.115826
For any particular negative test results, the probability that it is:			
True Negative	0.8	0.625358	0.909386
False Negative	0.2	0.090614	0.374642

Table B 6. Sensitivity and specificity for urines containing bacteria (A) and cultivated isolates from these urines (B) producing ESBLs for all tested cephalosporins.

(A) Urines containing bacteria producing ESBLs

Values entered:			
	Conditions		
	Urine S	**Urine R**	**Totals**
Cephalosporin hydrolysed	0	191	191
Cephalosporin not hydrolysed	0	18	18
Totals	0	210	210
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	1	0.977503	1
Sensitivity	0.913876	0.865181	0.946717
Specificity	-	-	-
For any particular test results, the probability that it will be:			
Positive	0.913876	0.865181	0.946171
Negative	0.086124	0.053283	0.134819
For any particular positive test results, the probability that it is:			
True Positive	1	0.975425	1
False Positive	0	0	0.0242575
For any particular negative test results, the probability that it is:			
True Negative	0	0	0.218756
False Negative	1	0.781244	1

(B) Cultivated isolates producing ESBLs

Values entered:			
	Conditions		
	Isolates S	**Isolates R**	**Totals**
Cephalosporin hydrolysed	0	207	207
Cephalosporin not hydrolysed	0	4	4
Totals	0	211	211
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	1	0.977712	1
Sensitivity	0.981043	0.948985	0.99391
Specificity	-	-	-
For any particular test results, the probability that it will be:			
Positive	0.981043	0.948985	0.99391
Negative	0.018957	0.00609	0.051015
For any particular positive test results, the probability that it is:			
True Positive	1	0.97729	1
False Positive	0	0	0.02271
For any particular negative test results, the probability that it is:			
True Negative	0	0	0.604226
False Negative	1	0.395774	1

Table B 7. Sensitivity and specificity for urines containing bacteria (A) and cultivated isolates from these urines (B) producing AmpC for all tested cephalosporins.

(A) Urines containing bacteria producing AmpC β-lactamases

Values entered:			
	Conditions		
	Urines S	**Urines R**	**Totals**
Cephalosporin hydrolysed	0	24	24
Cephalosporin not hydrolysed	0	50	50
Totals	0	74	74
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	1	0.93851	1
Sensitivity	0.324324	0.222763	0.444292
Specificity	-	-	-
For any particular test results, the probability that it will be:			
Positive	0.324324	0.222763	0.444292
Negative	0.675676	0.555708	0.777237
For any particular positive test results, the probability that it is:			
True Positive	1	0.828285	1
False Positive	0	0	0.171715
For any particular negative test results, the probability that it is:			
True Negative	0	0	0.088875
False Negative	1	0.911125	1

(B) Cultivated isolates producing AmpC β-lactamases

Values entered:			
	Conditions		
	Isolates S	**Isolates R**	**Totals**
Cephalosporin hydrolysed	0	51	51
Cephalosporin not hydrolysed	0	22	22
Totals	0	73	73
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	1	0.93771	1
Sensitivity	0.69863	0.578505	0.797641
Specificity	-	-	-
For any particular test results, the probability that it will be:			
Positive	0.69863	0.578505	0.797641
Negative	0.30137	0.202359	0.421495
For any particular positive test results, the probability that it is:			
True Positive	1	0.912744	1
False Positive	0	0	0.087256
For any particular negative test results, the probability that it is:			
True Negative	0	0	0.184975
False Negative	1	0.815025	1

Appendix C

Sensitivity and specificity for the 16-Plex assay

Table C 1. Sensitivity and specificity for detection of *bla*$_{TEM}$ from urines (A) and cultivated isolates (B).

Table C 2. Sensitivity and specificity for detection of *bla*$_{SHV}$ from urines (A) and cultivated isolates (B).

Table C 3. Sensitivity and specificity for detection of *bla*$_{CTX\text{-}Mgr1}$ from urines (A) and cultivated isolates (B).

Table C 4. Sensitivity and specificity for detection of *bla*$_{CTX\text{-}Mgr9}$ from urines (A) and cultivated isolates (B).

Table C 5. Sensitivity and specificity for detection of *bla*$_{CMY}$ from urines (A) and cultivated isolates (B).

Table C 6. Sensitivity and specificity for detection of *bla*$_{OXA\text{-}1}$ from urines (A) and cultivated isolates (B).

Table C 7. Sensitivity and specificity for detection of *bla*$_{OXA\text{-}48}$ from cultivated isolates.

Table C 8. Sensitivity and specificity for detection of *bla*$_{KPC}$ from cultivated isolates.

Table C 9. Sensitivity and specificity for detection of *bla*$_{NDM}$ from cultivated isolates.

Table C 10. Sensitivity and specificity for detection of *dfrA1/A5/A7/A12* from urines (A) and cultivated isolates (B).

Table C 11. Sensitivity and specificity for detection of *aac(6')-Ib* from urines (A) and cultivated isolates (B).

Table C 12. Sensitivity and specificity for detection of *aadA1/A2/A3* from cultivated isolates.

Table C 1. Sensitivity and specificity for detection of *bla*$_{TEM}$ from urines (A) and cultivated isolates (B).

(A) *bla*$_{TEM}$ found in urines

Values entered:			
	Conditions		
	rtPCR -ve	**rtPCR +ve**	**Totals**
MT-PCR +ve	2	31	33
MT-PCR -ve	41	0	42
Totals	43	31	85
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	0.418919	0.307065	0.539245
Sensitivity	1	0.86273	1
Specificity	0.953488	0.82944	0.991903
For any particular test results, the probability that it will be:			
Positive	0.445946	0.3319	0.565637
Negative	0.554054	0.434363	0.6681
For any particular positive test results, the probability that it is:			
True Positive	0.939394	0.783791	0.989433
False Positive	0.060606	0.010567	0.216209
For any particular negative test results, the probability that it is:			
True Negative	1	0.893306	1
False Negative	1	1	0.106694

(B) *bla*$_{TEM}$ found in cultivated isolates

Values entered:			
	Conditions		
	rtPCR -ve	**rtPCR +ve**	**Totals**
MT-PCR +ve	0	24	24
MT-PCR -ve	11	0	11
Totals	11	24	35
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	0.685714	0.505793	0.825686
Sensitivity	1	0.828285	1
Specificity	1	0.678553	1
For any particular test results, the probability that it will be:			
Positive	0.685714	0.505793	0.825686
Negative	0.314286	0.174314	0.494207
For any particular positive test results, the probability that it is:			
True Positive	1	0.828285	1
False Positive	0	0	0.171715
For any particular negative test results, the probability that it is:			
True Negative	1	0.678553	1
False Negative	0	0	0.321447

Table C 2. Sensitivity and specificity for detection of *bla*$_{SHV}$ from urines (A) and cultivated isolates (B).

(A) *bla*$_{SHV}$ found in urines

Values entered:			
	Conditions		
	rtPCR -ve	**rtPCR +ve**	**Totals**
MT-PCR +ve	0	6	6
MT-PCR -ve	68	0	68
Totals	68	6	74
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	0.81081	0.033391	0.174267
Sensitivity	1	0.516818	1
Specificity	1	0.933377	1
For any particular test results, the probability that it will be:			
Positive	0.081081	0.033391	0.174267
Negative	0.918919	0.825733	0.966609
For any particular positive test results, the probability that it is:			
True Positive	1	0.516818	1
False Positive	0	0	0.483182
For any particular negative test results, the probability that it is:			
True Negative	1	0.933377	1
False Negative	0	0	0.066623

(B) *bla*$_{SHV}$ found in cultivated isolates

Values entered:			
	Conditions		
	rtPCR -ve	**rtPCR +ve**	**Totals**
MT-PCR +ve	0	16	16
MT-PCR -ve	19	0	19
Totals	19	16	35
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	0.457143	0.292194	0.631265
Sensitivity	1	0.759265	1
Specificity	1	0.790795	1
For any particular test results, the probability that it will be:			
Positive	0.457143	0.292194	0.631265
Negative	0.542857	0.368735	0.7-7806
For any particular positive test results, the probability that it is:			
True Positive	1	0.759265	1
False Positive	0	0	0.240735
For any particular negative test results, the probability that it is:			
True Negative	1	0.790795	1
False Negative	0	0	0.209205

Table C 3. Sensitivity and specificity for detection of *bla*$_{CTX\text{-}Mgr1}$ dfrom urines (A) and cultivated isolates (B).

(A) *bla*$_{CTX\text{-}Mgr1}$ found in urines

Values entered:			
	Conditions		
	rtPCR -ve	**rtPCR +ve**	**Totals**
MT-PCR +ve	1	23	24
MT-PCR -ve	50	0	50
Totals	51	23	24
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	0.310811	0.211078	0.430378
Sensitivity	1	0.821902	1
Specificity	0.980392	0.882072	0.998976
For any particular test results, the probability that it will be:			
Positive	0.324324	0.222763	0.444292
Negative	0.675676	0.555708	0.777237
For any particular positive test results, the probability that it is:			
True Positive	0.958333	0.768838	0.997821
False Positive	0.041667	0.002179	0.231162
For any particular negative test results, the probability that it is:			
True Negative	1	0.911125	1
False Negative	0	0	0.088875

(B) *bla*$_{CTX\text{-}Mgr1}$ found in cultivated isolates

Values entered:			
	Conditions		
	rtPCR -ve	**rtPCR +ve**	**Totals**
MT-PCR +ve	0	18	18
MT-PCR -ve	17	0	17
Totals	17	18	35
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	0.514286	0.342758	0.682757
Sensitivity	1	0.781244	1
Specificity	1	0.770779	1
For any particular test results, the probability that it will be:			
Positive	0.514286	0.342758	0.682757
Negative	0.485714	0.317243	0.657242
For any particular positive test results, the probability that it is:			
True Positive	1	0.788244	1
False Positive	0	0	0.318756
For any particular negative test results, the probability that it is:			
True Negative	1	0.770779	1
False Negative	0	0	0.229221

Table C 4. Sensitivity and specificity for detection of *bla*$_{CTX\text{-}Mgr9}$ from urines (A) and cultivated isolates (B).

(A) *bla*$_{CTX\text{-}Mgr9}$ found in urines

Values entered:			
	Conditions		
	rtPCR -ve	**rtPCR +ve**	**Totals**
MT-PCR +ve	0	13	13
MT-PCR -ve	61	0	61
Totals	61	13	74
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	0.175676	0.100435	0.285317
Sensitivity	1	0.716557	1
Specificity	1	0.92619	1
For any particular test results, the probability that it will be:			
Positive	0.175676	0.100435	0.285317
Negative	0.824324	0.714683	0.899565
For any particular positive test results, the probability that it is:			
True Positive	1	0.716567	1
False Positive	0	0	0.899565
For any particular negative test results, the probability that it is:			
True Negative	1	0.92619	1
False Negative	0	0	0.07381

(B) *bla*$_{CTX\text{-}Mgr9}$ found in cultivated isolates

Values entered:			
	Conditions		
	rtPCR -ve	**rtPCR +ve**	**Totals**
MT-PCR +ve	0	4	4
MT-PCR -ve	31	0	31
Totals	31	4	35
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	0.114286	0.037256	0.176797
Sensitivity	1	0.395774	1
Specificity	1	0.86273	1
For any particular test results, the probability that it will be:			
Positive	0.114286	0.037256	0.276797
Negative	0.885714	0.723203	0.962744
For any particular positive test results, the probability that it is:			
True Positive	1	0.395744	1
False Positive	0	0	0.604226
For any particular negative test results, the probability that it is:			
True Negative	1	0.86273	1
False Negative	0	0	0.13727

Table C 5. Sensitivity and specificity for detection of *bla*$_{CMY}$ from urines (A) and cultivated isolates (B).

(A) *bla*$_{CMY}$ found in urines

Values entered:			
	Conditions		
	rtPCR -ve	**rtPCR +ve**	**Totals**
MT-PCR +ve	0	9	9
MT-PCR -ve	65	0	65
Totals	65	9	74
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	0.121622	0.060532	0.223258
Sensitivity	1	0.628811	1
Specificity	1	0.930476	1
For any particular test results, the probability that it will be:			
Positive	0.121622	0.060532	0.223258
Negative	0.878378	0.776742	0.939468
For any particular positive test results, the probability that it is:			
True Positive	1	0.628811	1
False Positive	0	0	0.371189
For any particular negative test results, the probability that it is:			
True Negative	1	0.930476	1
False Negative	0	0	0.069524

(B) *bla*$_{CMY}$ found in cultivated isolates

Values entered:			
	Conditions		
	rtPCR -ve	**rtPCR +ve**	**Totals**
MT-PCR +ve	0	7	7
MT-PCR -ve	28	0	28
Totals	28	7	35
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	0.2	0.090614	0.374642
Sensitivity	1	0.560935	1
Specificity	1	0.848819	1
For any particular test results, the probability that it will be:			
Positive	0.2	0.090614	0.374642
Negative	0.8	0.625358	0.909386
For any particular positive test results, the probability that it is:			
True Positive	1	0.560935	1
False Positive	0	0	0.439065
For any particular negative test results, the probability that it is:			
True Negative	1	0.849819	1
False Negative	0	0	0.150181

Table C 6. Sensitivity and specificity for detection of *bla*$_{OXA-1}$ from urines (A) and cultivated isolates (B).

(A) *bla*$_{OXA-1}$ found in urines

Values entered:			
	Conditions		
	rtPCR -ve	**rtPCR +ve**	**Totals**
MT-PCR +ve	0	8	8
MT-PCR -ve	66	0	66
Totals	66	8	74
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	0.108108	0.051155	0.20721
Sensitivity	1	0.597704	1
Specificity	1	0.931471	1
For any particular test results, the probability that it will be:			
Positive	0.108108	0.051155	0.20721
Negative	0.891892	0.931471	1
For any particular positive test results, the probability that it is:			
True Positive	1	0.597704	1
False Positive	0	0	0.402296
For any particular negative test results, the probability that it is:			
True Negative	1	0.931371	1
False Negative	0	O	0.068529

(B) *bla*$_{OXA-1}$ found in cultivated isolates

Values entered:			
	Conditions		
	rtPCR -ve	**rtPCR +ve**	**Totals**
MT-PCR +ve	0	19	19
MT-PCR -ve	16	0	16
Totals	16	19	35
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	0.542857	0.368735	0.707806
Sensitivity	1	0.790795	1
Specificity	1	0.759265	1
For any particular test results, the probability that it will be:			
Positive	0.542857	0.368735	0.707806
Negative	0.457143	0.292194	0.631265
For any particular positive test results, the probability that it is:			
True Positive	1	0.790795	1
False Positive	0	0	0.209205
For any particular negative test results, the probability that it is:			
True Negative	1	0.759265	1
False Negative	0	0	0.240735

Table C 7. Sensitivity and specificity for detection of *bla*$_{\text{OXA-48}}$ from cultivated isolates.

Values entered:			
	Conditions		
	rtPCR -ve	**rtPCR +ve**	**Totals**
MT-PCR +ve	0	7	7
MT-PCR -ve	28	0	28
Totals	28	7	35
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	0.2	0.090614	0.374642
Sensitivity	1	0.560935	1
Specificity	1	0.849819	1
For any particular test results, the probability that it will be:			
Positive	0.2	0.090614	0.374642
Negative	0.8	0.625358	0.909386
For any particular positive test results, the probability that it is:			
True Positive	1	0.560935	1
False Positive	0	0	0.439065
For any particular negative test results, the probability that it is:			
True Negative	1	0.849819	1
False Negative	0	0	0.150181

Table C 8. Sensitivity and specificity for detection of *bla*$_{\text{KPC}}$ from cultivated isolates.

Values entered:			
	Conditions		
	rtPCR -ve	**rtPCR +ve**	**Totals**
MT-PCR +ve	0	5	5
MT-PCR -ve	30	0	30
Totals	30	5	35
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	0.142857	0.053815	0.310421
Sensitivity	1	0.462945	1
Specificity	1	0.85868	1
For any particular test results, the probability that it will be:			
Positive	0.142857	0.053815	0.310421
Negative	0.857143	0.689579	0.946185
For any particular positive test results, the probability that it is:			
True Positive	1	0.462945	1
False Positive	0	0	0.537055
For any particular negative test results, the probability that it is:			
True Negative	1	0.85868	1
False Negative	0	0	0.14132

Table C 9. Sensitivity and specificity for detection of *bla*$_{NDM}$ from cultivated isolates.

Values entered:			
	Conditions		
	rtPCR -ve	**rtPCR +ve**	**Totals**
MT-PCR +ve	0	10	10
MT-PCR -ve	25	0	25
Totals	25	10	35
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	0.285714	0.152369	0.465246
Sensitivity	1	0.655464	1
Specificity	1	0.834227	1
For any particular test results, the probability that it will be:			
Positive	0.285714	0.152369	0.465246
Negative	0.714286	0.534754	0.847631
For any particular positive test results, the probability that it is:			
True Positive	1	0.655464	1
False Positive	0	0	0.344536
For any particular negative test results, the probability that it is:			
True Negative	1	0.834227	1
False Negative	0	0	0.165773

Table C 10. Sensitivity and specificity for detection of *dfrA1/A5/A7/A12* from urines (A) and cultivated isolates (B).

(A) *dfrA1/A5/A7/A12* found in urines

Values entered:			
	Conditions		
	Trim S	**Trim R**	**Totals**
MT-PCR +ve	1	38	39
MT-PCR -ve	32	3	35
Totals	33	41	74
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	0.554054	0.434363	0.6681
Sensitivity	0.926829	0.789948	0.98091
Specificity	0.969697	0.824868	0.998416
For any particular test results, the probability that it will be:			
Positive	0.527027	0.408283	0.642948
Negative	0.472973	0.357051	0.591717
For any particular positive test results, the probability that it is:			
True Positive	0.974359	0.84924	0.99866
False Positive	0.025641	0.00134	0.15076
For any particular negative test results, the probability that it is:			
True Negative	0.914286	0.758136	0.977598
False Negative	0.085714	0.022402	0.241864

(B) *dfrA1/A5/A7/A12* found in isolates

Values entered:			
	Conditions		
	Trim S	**Trim R**	**Totals**
MT-PCR +ve	0	30	30
MT-PCR -ve	3	2	5
Totals	3	32	35
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	0.914286	0.758136	0.977598
Sensitivity	0.9375	0.777848	0.9891
Specificity	1	0.309989	1
For any particular test results, the probability that it will be:			
Positive	0.857143	0.689579	0.946185
Negative	0.142857	0.053815	0.310421
For any particular positive test results, the probability that it is:			
True Positive	1	0.95868	1
False Positive	0	0	0.14132
For any particular negative test results, the probability that it is:			
True Negative	0.6	0.170424	0.927416
False Negative	0.4	0.072584	0.829576

Table C 11. Sensitivity and specificity for detection of *aac(6')-Ib* from urines (A) and cultivated isolates (B).

(A) *aac(6')-Ib* found in urines

Values entered:			
	Conditions		
	Tobra S	**Tobra R**	**Totals**
MT-PCR +ve	1	8	9
MT-PCR -ve	65	0	65
Totals	66	8	74
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	0.108108	0.051155	0.20721
Sensitivity	1	0.597704	1
Specificity	0.984848	0.907309	0.999209
For any particular test results, the probability that it will be:			
Positive	0.121622	0.060532	0.223258
Negative	0.878378	0.776742	0.939468
For any particular positive test results, the probability that it is:			
True Positive	0.888889	0.506703	0.994172
False Positive	0.111111	0.005828	0.493297
For any particular negative test results, the probability that it is:			
True Negative	1	0.930476	1
False Negative	0	0	0.069524

(B) *aac(6')-Ib* found in isolates

Values entered:			
	Conditions		
	Tobra S	**Tobra R**	**Totals**
MT-PCR +ve	0	22	22
MT-PCR -ve	11	2	13
Totals	11	24	35
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	0.685714	0.505793	0.825686
Sensitivity	0.916667	0.715289	0.985433
Specificity	1	0.678553	1
For any particular test results, the probability that it will be:			
Positive	0.628571	0.449486	0.780077
Negative	0.371429	0.219923	0.550514
For any particular positive test results, the probability that it is:			
True Positive	1	0.815025	1
False Positive	0	0	0.184975
For any particular negative test results, the probability that it is:			
True Negative	0.846154	0.536625	0.971893
False Negative	0.153846	0.027107	0.463375

Table C 12. Sensitivity and specificity for detection of *aadA1/A2/A3* from urines (A) and cultivated isolates (B).

(A) *aadA1/A2/A3* found in urines

Values entered:			
	Conditions		
	Strep S	**Strep R**	**Totals**
MT-PCR +ve	7	9	16
MT-PCR -ve	43	15	58
Totals	50	24	74
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	0.324324	0.222763	0.444292
Sensitivity	0.375	0.195502	0.592424
Specificity	0.86	0.726437	0.93723
For any particular test results, the probability that it will be:			
Positive	0.216216	0.132271	0.330116
Negative	0.783784	0.669884	0.867729
For any particular positive test results, the probability that it is:			
True Positive	0.5625	0.305543	0.792462
False Positive	0.4375	0.207538	0.694457
For any particular negative test results, the probability that it is:			
True Negative	0.741379	0.607066	0.843504
False Negative	0.4375	0.207538	0.694457

(B) *aadA1/A2/A3* found in isolates

Values entered:			
	Conditions		
	Strep S	**Strep R**	**Totals**
MT-PCR +ve	6	10	16
MT-PCR -ve	11	8	19
Totals	17	18	45
	Estimated value	**95% Confidence interval**	
		Lower limit	**Upper limit**
Prevalence	0.514286	0.342758	0.682757
Sensitivity	0.555556	0.31347	0.775952
Specificity	0.647059	0.386204	0.847407
For any particular test results, the probability that it will be:			
Positive	0.457143	0.292194	0.631265
Negative	0.542857	0.368735	0.707806
For any particular positive test results, the probability that it is:			
True Positive	0.625	0.358736	0.837163
False Positive	0.375	0.162837	0.641241
For any particular negative test results, the probability that it is:			
True Negative	0.578947	0.3396790	0.788793
False Negative	0.421053	0.211207	0.660321

Appendix D

Quality and quantity of the extracted DNA from Clinical Urines 1-10 and Spiked Urines used for the MinION sequencing

Figure D 1. The quality and quantity of the depleted DNA using Tape Station gel analysis and Qubit for all sequences Clinical and Spiked Urines.

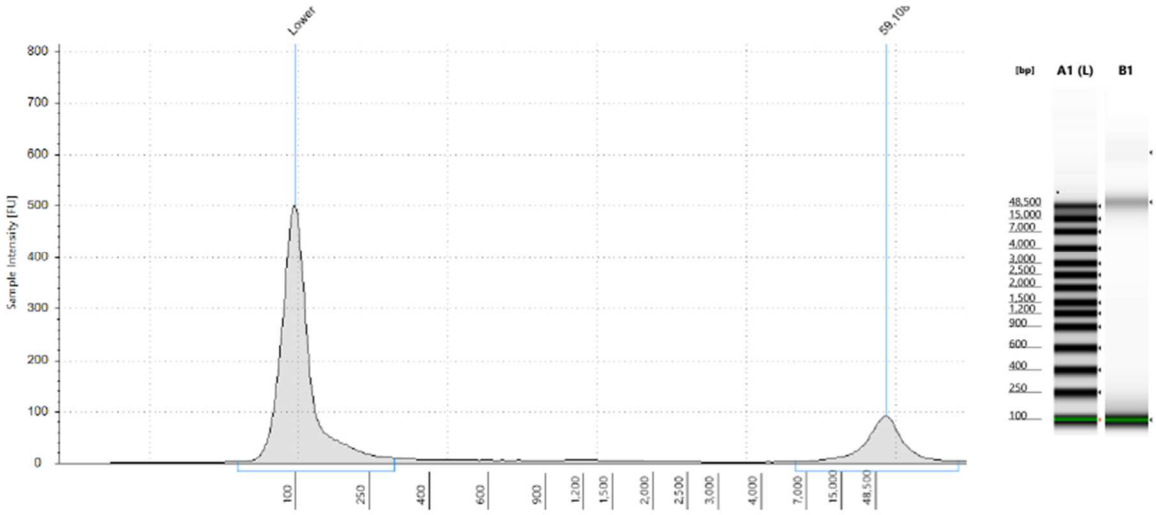
Table D 1. Number of BLAST hits and contings that matched *proteobacterial* genomes for all sequenced Clinical and Spiked Urines using MinION and Illumina sequencing.

Figure D 1. The quality and quantity of the depleted DNA using Tape Station gel analysis and Qubit for all sequenced Clinical and Spiked Urines.

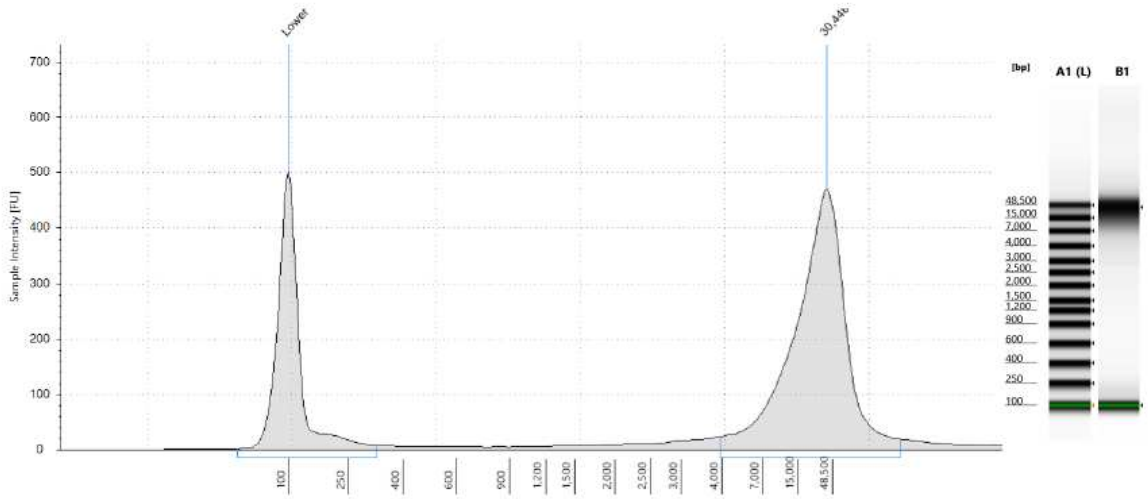
Legend: SU Spiked Urine; CU Clinical Urine; SCU Spiked Clinical Urine; lines A1-ladder; B1- DNA extracted from urine according to the methodology described in Section 2.4. Samples CU1 and 2 were not run on the tape station; CU3- Lack of bands (48500bp) in the line B1 due to extraction from frozen urine.

(A)- CU4; (B)- SURun1; (C)- CU5; (D)- CU6; (E)- CU7; (F)- SCU6; (G)- CU8; (H)- CU9; (I)- CU10; (J)-SURun2; (K)- SURun3; (L)- SURun4.

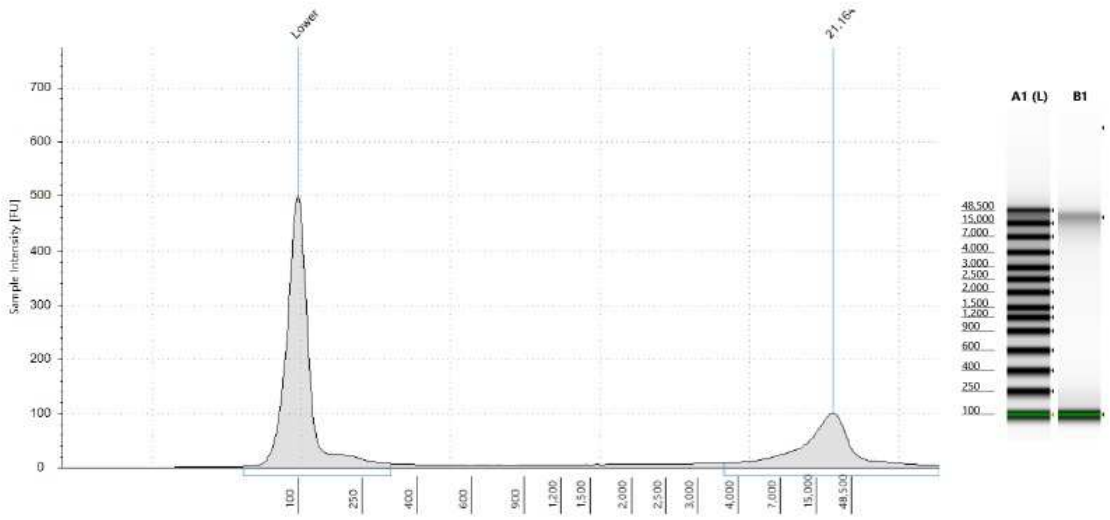
(A) Clinical Urine 4; DNA concentration= 8 ng/µl



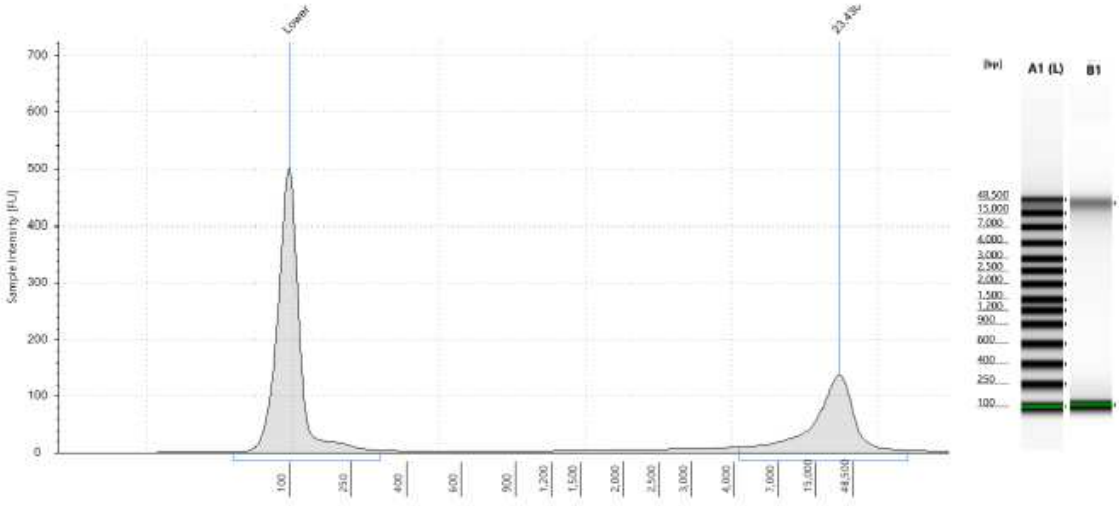
(B) Spiked Urine Run1; DNA concentration = 85.6 ng/µl



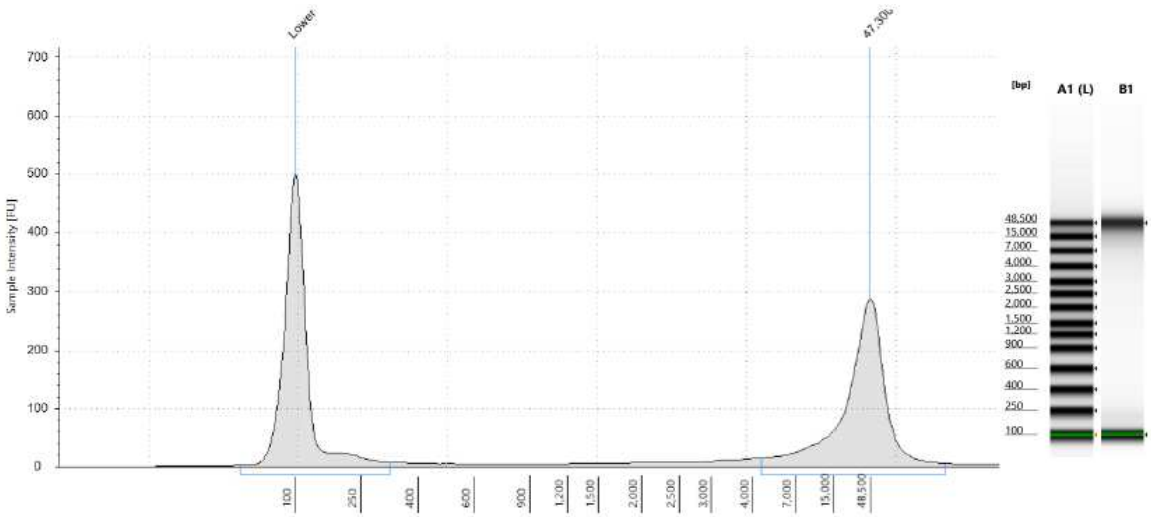
(C) Clinical Urine 5; DNA concentration = 35.5 ng/µl



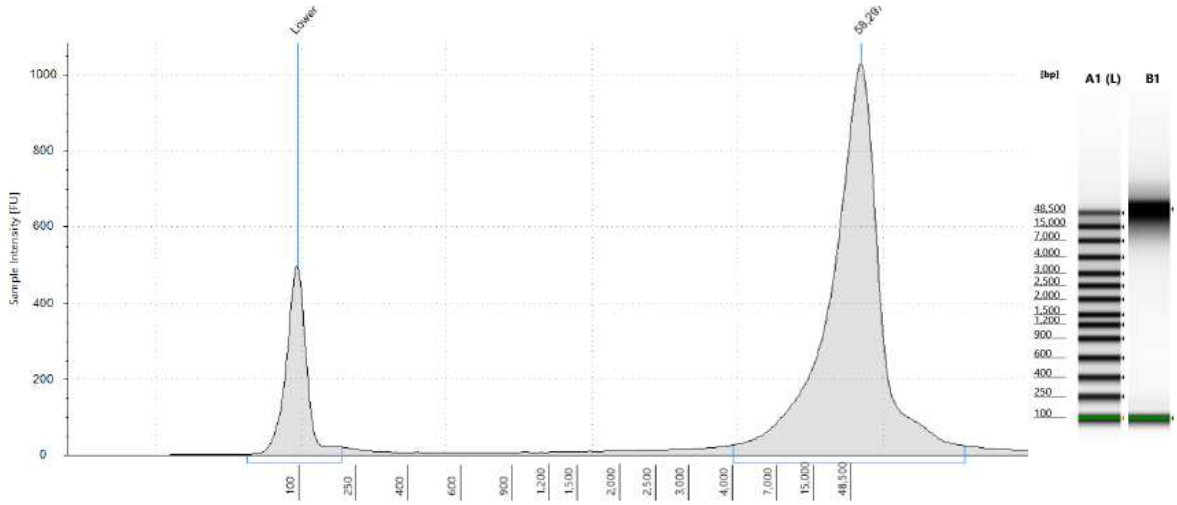
(D) Clinical Urine 6; DNA concentration = 8.12 ng/µl



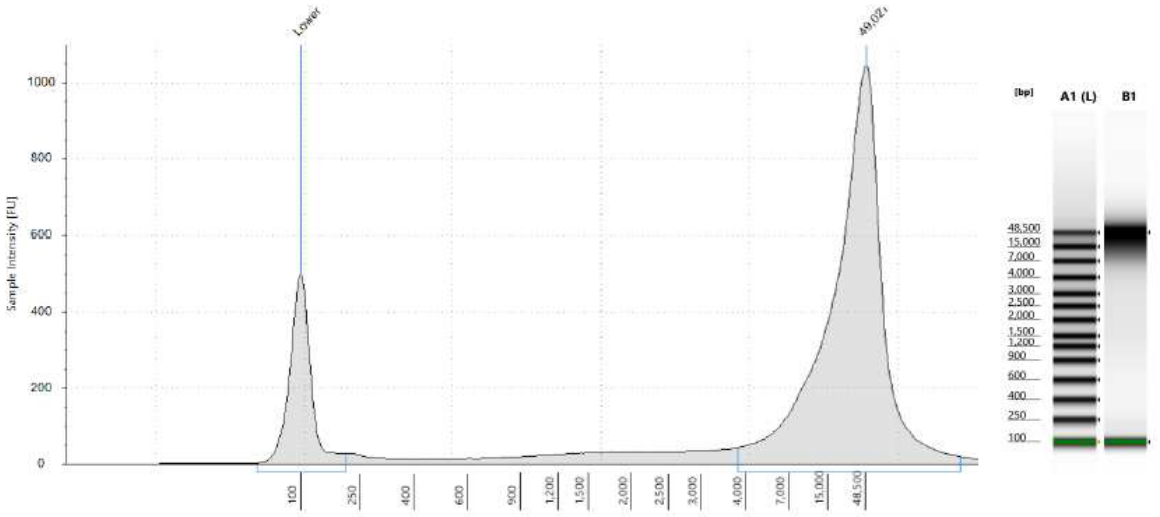
(E) Clinical Urine 7; DNA concentration= 42.6 ng/µl



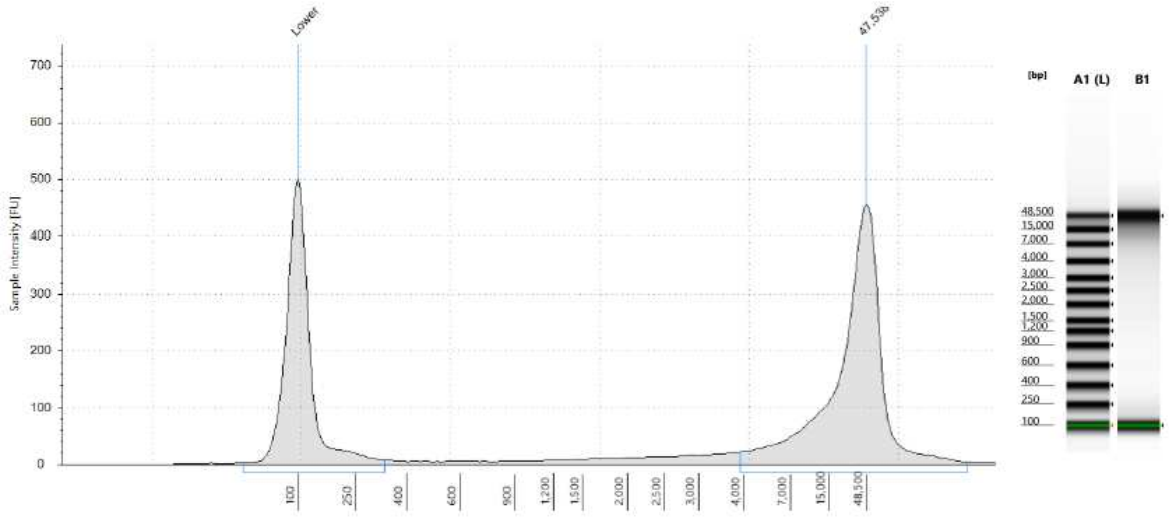
(F) Spiked Clinical Urine 6; DNA concentration= 104 ng/µl



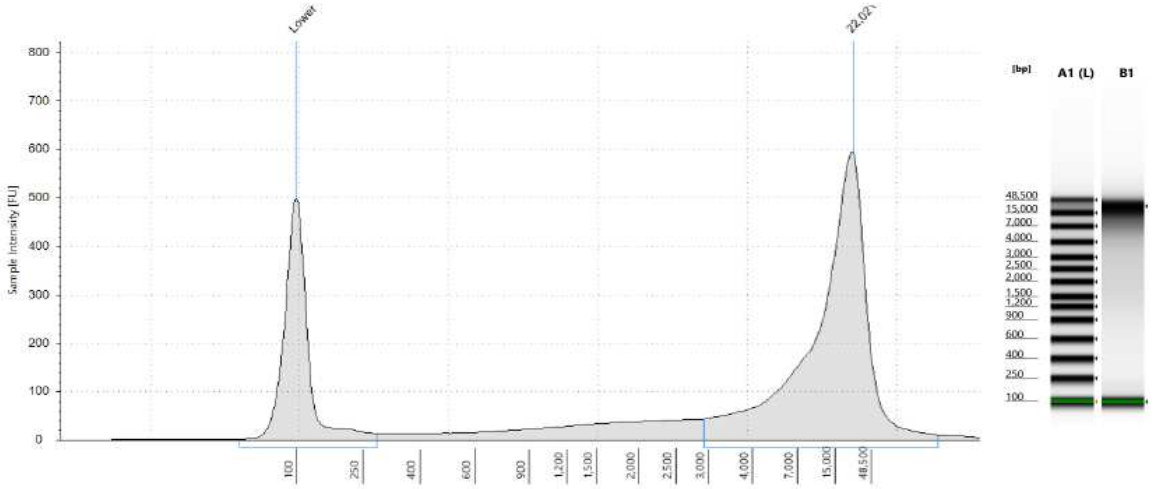
(G) Clinical Urine 8; DNA concentration= 62.22 ng/µl



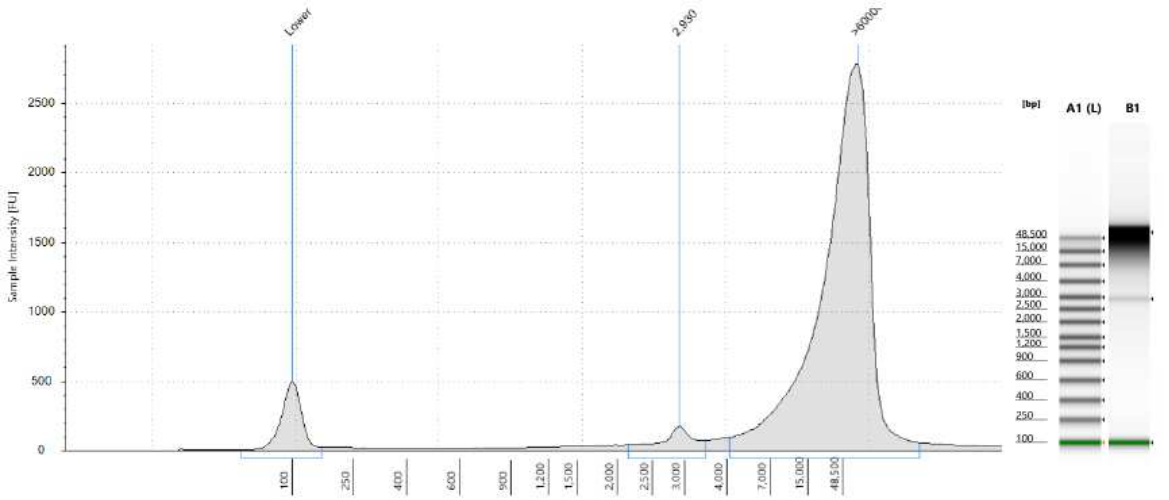
(H) Clinical Urine 9; DNA concentration= 31.4 ng/µl



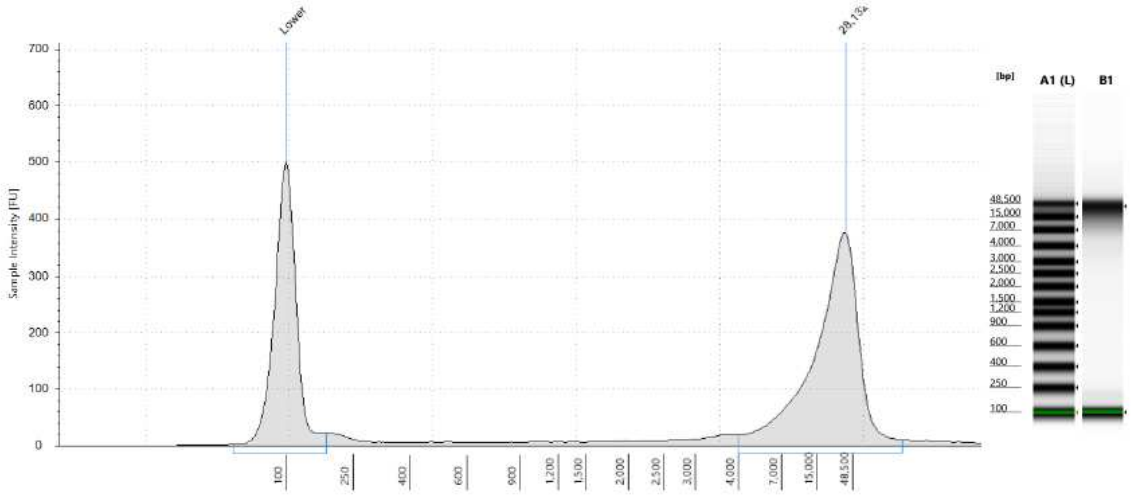
(I) Clinical Urines 10; DNA concentration= 53.9 ng/µl



(J) Spiked Urine Run 2; DNA concentration= 102 ng/µl



(K) Spiked Urine Run 3; DNA concentration= 67.8 ng/µl



(L) Spiked Urine Run 4; DNA concentration= 61.2 ng/µl

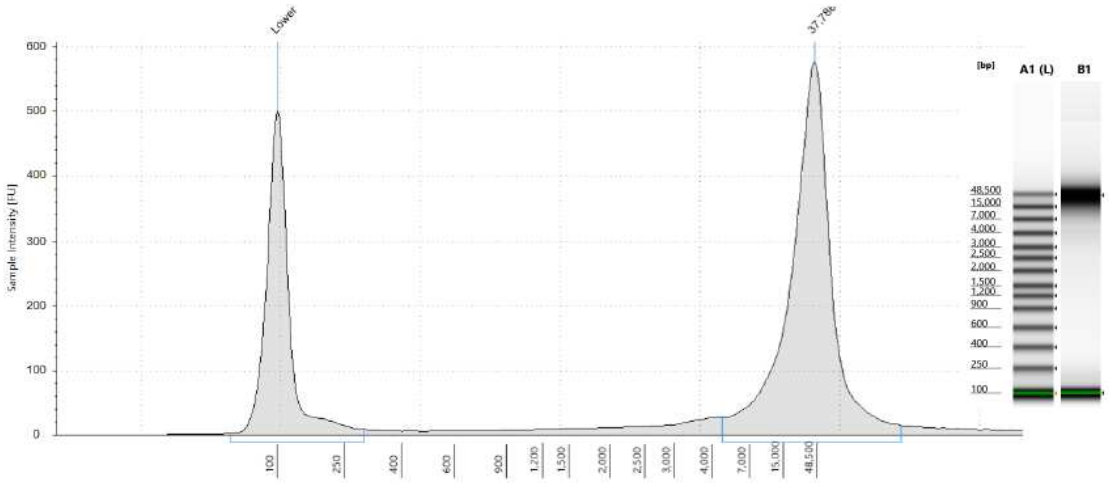


Table D 1. Number of BLAST hits and contings that matched *proteobacterial* genomes for all sequenced Clinical and Spiked Urines using MinION and Illumina sequencing.

Clinical urine 5			
MinION		**Illumina**	
Hits	**Proteobacterial genomes**	**Contigs**	**Proteobacterial genomes**
1	*Acetobacter pasteurianus*	1	*Enterobacter aerogenes*
1	*Acinetobacter baumannii*	1	*Salmonella enterica*
1	*Acinetobacter haemolyticus*	1	*Shigella flexneri*
1	*Azoarcus* sp.	1	*uncultured Klebsiella*
1	*Azospirillum lipoferum*	2	*Salmonella* sp.
1	*Azotobacter vinelandii*	4	*Klebsiella oxytoca*
1	*Brevundimonas subvibrioides*	14	*Escherichia coli*
1	*Chromobacterium* sp.	72	*Klebsiella pneumoniae*
1	*Delftia* sp.		
1	*Desulfarculus baarsii*		
1	*Desulfovibrio desulfuricans*		
1	*Edwardsiella tarda*		
1	*Enterobacter cloacae*		
1	*Enterobacter lignolyticus*		
1	*Ferrimonas balearica*		
1	*Helicobacter pylori*		
1	*Leptothrix cholodnii*		
1	*Methylibium petroleiphilum*		
1	*Pseudomonas mendocina*		
1	*Pseudomonas putida*		
1	*Pseudomonas* sp.		
1	*Rhizobium etli*		
1	*Saccharophagus degradans*		
1	*Salmonella bongori*		
1	*Sinorhizobium medicae*		
1	*Sinorhizobium meliloti*		
1	*Stenotrophomonas maltophilia*		
1	*Syntrophus aciditrophicus*		
1	*Thauera* sp.		
1	*Yersinia pestis*		
2	*Escherichia fergusonii*		
2	*Klebsiella variicola*		
3	*Raoultella ornithinolytica*		
4	*Salmonella enterica*		
5	*Escherichia coli*		
6	*Klebsiella oxytoca*		
7	*1 phylum*		
7	*Enterobacter aerogenes*		
1881	*Klebsiella pneumonia*		

Top Hits	**Supgroup**	**Contigs**	**Supgroup**
464	*K. pneumoniae* CG43	954	*K. pneumoniae* MGH 78578
338	*K. pneumoniae* JM45	421	*K. pneumoniae* CG43
266	*K. pneumoniae* MGH 78578	167	*K. pneumoniae* JM45
Clinical urine 6			
MinION		**Illumina**	
Hits	**Proteobacterial genomes**	**Contigs**	**Proteobacterial genomes**
1	*Aeromonas caviae*	1	*Alteromonas macleodii*
1	*Enterobacter hormaechei*	1	*Enterobacter cloacae*
1	*Enterobacter lignolyticus*	1	*Shigella boydii*
1	*Pantoea* sp.	1	*Shigella flexneri*
1	*Vibrio cholerae*	2	*Salmonella enterica*
1	*uncultured Enterobacteriaceae*	2	*Shigella sonnei*
1	*uncultured Shigella*	5	*Klebsiella pneumoniae*
2	*1 phylum*	177	*Escherichia coli*
2	*Pectobacterium* sp.		
2	*Yersinia pestis*		
3	*Enterobacter* sp.		
4	*Citrobacter rodentium*		
6	*Shigella boydii*		
6	*Shigella dysenteriae*		
10	*Escherichia fergusonii*		
11	*Klebsiella oxytoca*		
14	*Enterobacter cloacae*		
16	*Shigella flexneri*		
25	*Shigella sonnei*		
81	*Klebsiella pneumoniae*		
93	*Salmonella enterica*		
11697	*Escherichia coli*		
Top Hits	**Supgroup**	**Contigs**	**Supgroup**
9495	*Escherichia coli* JJ1886	3319	*Escherichia coli* JJ1886
281	*Escherichia coli* NA114	209	*Escherichia coli* SE15 DNA
166	*Escherichia coli* UM146	49	*Escherichia coli* O111:H-str. 11128
Clinical urine 7			
MinION		**Illumia**	
Hits	**Proteobacterial genomes**	**Contigs**	**Proteobacterial genomes**
1	*Acidithiobacillus ferrooxidans*	1	*Klebsiella oxytoca*
1	*Acinetobacter baumannii*	1	*Providencia stuartii*
1	*Acinetobacter baylyi*	2	*Shigella sonnei*
1	*Aeromonas hydrophila*	10	*Salmonella enterica*
1	*Alcanivorax dieselolei*	11	*Klebsiella pneumoniae*
1	*Bradyrhizobium* sp.	139	*Escherichia coli*
1	*Brenneria nigrifluens*		
1	*Candidatus Blochmannia*		
1	*Candidatus Legionella*		
1	*Citrobacter freundii*		
1	*Desulfocapsa sulfexigens*		
1	*Dickeya dadantii*		
1	*Gluconobacter oxydans*		

MinION			
Hits	**Proteobacterial genomes**		
1	*Haliangium ochraceum*		
1	*Histophilus somni*		
1	*Hyphomicrobium denitrificans*		
1	*Klebsiella variicola*		
1	*Nilaparvata lugens*		
1	*Ochrobactrum thiophenivorans*		
1	*Pantoea ananatis*		
1	*Pasteurella aerogenes*		
1	*Pseudomonas aeruginosa*		
1	*Pseudomonas fluorescens*		
1	*Pseudomonas putida*		
1	*Pseudovibrio* sp.		
1	*Psychrobacter* sp.		
1	*Salmonella bongori*		
1	*Shewanella oneidensis*		
1	*Sphingopyxis alaskensis*		
1	*Stenotrophomonas sp.*		
1	*Teredinibacter turnerae*		
1	*Tolumonas auensis*		
1	*Xanthobacter autotrophicus*		
1	*uncultured Desulfobacterium*		
1	*uncultured Enterobacteriaceae*		
1	*uncultured Shigella*		
2	*Haemophilus influenzae*		
2	*Moraxella catarrhalis*		
2	*Shigella* sp.		
3	*Citrobacter koseri*		
3	*Citrobacter rodentium*		
3	*Yersinia pestis*		
4	*Enterobacter hormaechei*		
4	*Proteus mirabilis*		
5	*Shigella dysenteriae*		
7	*Providencia stuartii*		
8	*Morganella morganii*		
9	*Klebsiella oxytoca*		
12	*Escherichia fergusonii*		
18	*Shigella boydii*		
29	*Shigella flexneri*		
33	*Shigella sonnei*		
46	*Klebsiella pneumoniae*		
123	*Salmonella enterica*		
8166	*Escherichia coli*		
Top Hits	**Supgroup**	**Contigs**	**Supgroup**
1235	*Escherichia coli* PMV-1 main chromosome	912	*Escherichia coli* IHE3034
1166	*Escherichia coli* str. 'clone D i 14' chromosome	553	*Escherichia coli* LF82

Top Hits	**Supgroup**	**Contigs**	**Supgroup**
838	*Escherichia coli* O83:H1 str.NRG 857C	491	*Escherichia coli* str. 'clone D i14'
Clinical urine 8			
MinION		**Illumina**	
Hits	**Proteobacterial genomes**	**Contigs**	**Proteobacterial genomes**
1	*Cronobacter sakazakii*	1	*Enterobacter aerogenes*
1	*Delftia* sp.	1	*Klebsiella oxytoca*
1	*Enterobacter lignolyticus*	1	*Marinomonas mediterranea*
1	*Enterobacteriaceae bacterium*	1	*Pasteurella multocida*
1	*Halomonas* sp.	1	*Pseudomonas aeruginosa*
1	*Pasteurella multocida*	1	*Salmonella enterica*
1	*Pelobacter propionicus*	1	*Yersinia enterocolitica*
1	*Pseudomonas mendocina*	1	uncultured proteobacterium
1	*Stenotrophomonas maltophilia*	2	*Enterobacter cloacae*
1	*Stx2-converting phage*	3	*Klebsiella pneumoniae*
1	*Thioalkalivibrio nitratireducens*	3	uncultured gamma
1	*Thioalkalivibrio* sp.	118	*Escherichia coli*
1	*secondary endosymbiont*		
1	*uncultured Shigella*		
2	*Citrobacter rodentium*		
3	*1 phylum*		
3	*Citrobacter koseri*		
4	*Pseudomonas aeruginosa*		
7	*Yersinia pestis*		
9	*Shigella boydii*		
11	*Enterobacter aerogenes*		
14	*Shigella dysenteriae*		
25	*Escherichia fergusonii*		
29	*Yersinia enterocolitica*		
31	*Shigella flexneri*		
35	*Shigella sonnei*		
59	*Klebsiella pneumoniae*		
100	*Salmonella enterica*		
16698	*Escherichia coli*		
Top Hits	**Supgroup**	**Contigs**	**Supgroup**
10112	*Escherichia coli* 536	3691	*Escherichia coli* 536
1110	*Escherichia coli* PMV-1	213	*Escherichia coli* JJ1886
929	*Escherichia coli* str. 'clone D i14'	107	*Escherichia coli* str. 'clone D i14'
Clinical urine 9			
MinION		**Illumina**	
Hits	**Proteobacterial genomes**	**Contigs**	**Proteobacterial genomes**
1	*Alicycliphilus denitrificans*	1	*Agrobacterium tumefaciens*
1	*Delftia acidovorans*	1	*Citrobacter koseri*
1	*Edwardsiella piscicida*	1	*Desulfomonile tiedjei*
1	*Enterobacter cancerogenus*	1	*Enterobacteriaceae*

			bacterium
MinION		**Illumina**	
Hits	**Proteobacterial genomes**	**Contigs**	**Proteobacterial genomes**
1	*Erwinia amylovora*	1	*Erwinia tasmaniensis*
1	*Klebsiella* sp.	1	*Neorickettsia risticii*
1	*Photobacterium profundum*	1	*Raoultella ornithinolytica*
1	*Proteus vulgaris*	1	*Serratia marcescens*
1	*Providencia stuartii*	1	*Shigella flexneri*
1	*Serratia proteamaculans*	1	*endosymbiont of uncultured Klebsiella*
1	*uncultured Citrobacter*		
2	*Citrobacter koseri*	1	*Enterobacter asburiae*
2	*Enterobacter lignolyticus*	1	*2Enterobacter* sp.
2	*Pectobacterium carotovorum*	4	*Cronobacter sakazakii*
3	*Cronobacter turicensis*	4	*Klebsiella pneumoniae*
3	*Erwinia tasmaniensis*	4	*Salmonella enterica*
3	*Pantoea agglomerans*	8	*Escherichia coli*
4	*Citrobacter rodentium*	52	*Enterobacter cloacae*
4	*Delftia* sp.		
4	*Klebsiella oxytoca*		
4	*Shigella flexneri*		
4	*Shigella sonnei*		
5	*Pseudomonas putida*		
6	*Enterobacteriaceae bacterium*		
10	*Serratia marcescens*		
16	*Enterobacter* sp.		
17	*Enterobacter hormaechei*		
19	*Enterobacter aerogenes*		
30	*Shimwellia blattae*		
38	*Cronobacter sakazakii*		
38	*Enterobacter asburiae*		
74	*Salmonella enterica*		
94	*Klebsiella pneumoniae*		
95	*Escherichia coli*		
5070	*Enterobacter cloacae*		
Top Hits	**Supgroup**	**Contigs**	**Supgroup**
10302	*Enterobacter cloacae* NCTC 9394	2996	*Enterobacter cloacae* NCTC 9394
539	*Enterobacter cloacae* ENHKU01	1157	*Enterobacter cloacae* ENHKU01
397	*Enterobacter cloacae* subsp. cloacae ATCC 13047	772	*Enterobacter cloacae* subsp. cloacae ATCC 13047
Clinical urine 10			
MinION		**Illumina**	
Hits	**Proteobacterial genomes**	**Contigs**	**Proteobacterial genomes**
1	*Alicycliphilus denitrificans*	1	*Enterobacter aerogenes*
1	*Azotobacter vinelandii*	1	*Enterobacter cloacae*
1	*Bibersteinia trehalosi*	1	*Klebsiella variicola*
1	*Bordetella petrii*	1	*Shigella flexneri*
1	*Bradyrhizobium* sp.	2	*Klebsiella oxytoca*
1	*Brenneria salicis*	3	*Escherichia coli*

MinION		Illumina	
Hits	**Proteobacterial genomes**	**Contigs**	**Proteobacterial genomes**
1	*Chromobacterium violaceum*	134	*Klebsiella pneumoniae*
1	*Citrobacter freundii*		
1	*Citrobacter koseri*		
1	*Citrobacter* sp.		
1	*Cloning vector*		
1	*Desulfovibrio vulgaris*		
1	*Edwardsiella piscicida*		
1	*Erwinia amylovora*		
1	*Helicobacter pylori*		
1	*Hyphomicrobium denitrificans*		
1	*Oligotropha carboxidovorans*		
1	*Pantoea* sp.		
1	*Pectobacterium atrosepticum*		
1	*Pectobacterium carotovorum*		
1	*Photobacterium damselae*		
1	*Proteus vulgaris*		
1	*Providencia rettgeri*		
1	*Pseudomonas denitrificans*		
1	*Pseudomonas mendocina*		
1	*Pseudomonas resinovorans*		
1	*Pseudomonas stutzeri*		
1	*Pseudoxanthomonas spadix*		
1	*Psychrobacter* sp.		
1	*Rahnella* sp.		
1	*Ramlibacter tataouinensis*		
1	*Rhodopseudomonas palustris*		
1	*Serratia proteamaculans*		
1	*Shuttle vector*		
1	*Simiduia agarivorans*		
1	*Sphingobium* sp.		
1	*Tistrella mobilis*		
1	*Xanthomonas euvesicatoria*		
1	*Yersinia enterocolitica*		
1	*Yersinia* sp.		
1	*uncultured Enterobacter*		
2	*Acidovorax ebreus*		
2	*Aeromonas hydrophila*		
2	*Shimwellia blattae*		
2	*Vibrio cholerae*		
2	*Yersinia pestis*		
3	*Bordetella bronchiseptica*		
3	*Cronobacter turicensis*		
3	*Dickeya dadantii*		
3	*Edwardsiella tarda*		
3	*Enterobacter* sp.		
3	*Pseudomonas fluorescens*		
3	*Pseudomonas* sp.		
3	*Rahnella aquatilis*		

MinION			
Hits	**Proteobacterial genomes**		
3	*Saccharophagus degradans*		
3	*Salmonella bongori*		
4	*Acinetobacter baumannii*		
4	*Aeromonas caviae*		
4	*Serratia marcescens*		
4	*Shigella boydii*		
4	*Shigella dysenteriae*		
4	*Sodalis glossinidius*		
4	*uncultured Klebsiella*		
5	*Achromobacter xylosoxidans*		
5	*Enterobacter lignolyticus*		
7	*Enterobacter asburiae*		
9	*Citrobacter rodentium*		
9	*Enterobacter hormaechei*		
9	*Vibrio furnissii*		
10	*Klebsiella* sp.		
12	*Raoultella ornithinolytica*		
20	*Cronobacter sakazakii*		
20	*Pseudomonas aeruginosa*		
21	*Shigella flexneri*		
33	*Stenotrophomonas maltophilia*		
34	*Delftia acidovorans*		
34	*Klebsiella oxytoca*		
37	*Enterobacter cloacae*		
52	*Delftia* sp.		
91	*Enterobacter aerogenes*		
132	*Klebsiella variicola*		
240	*Salmonella enterica*		
487	*Pseudomonas putida*		
949	*Escherichia coli*		
21349	*Klebsiella pneumoniae*		
Top Hits	**Supgroup**	**Contigs**	**Subgroup**
10549	*Klebsiella pneumoniae* CG43	650	*Klebsiella pneumoniae* MGH 78578
7241	*Klebsiella pneumoniae* JM45	614	*Klebsiella pneumoniae* CG43
6782	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578	424	*Klebsiella pneumoniae* NTUH-K2044
Spiked Clinical Urine 6			
MinION			
Hits	**Proteobacterial genomes**		
1	*Acinetobacter baumannii*		
1	*Citrobacter rodentium*		
1	*Enterobacter* sp.		
1	*Pseudomonas putida*		
1	*Yersinia pestis*		
2	*Escherichia sp.*		
2	*Shigella dysenteriae*		

3	*Klebsiella oxytoca*	
4	*Shigella flexneri*	
7	*Escherichia fergusonii*	
7	*Shigella boydii*	
9	*Enterobacter cloacae*	
16	*Shigella sonnei*	
30	*Klebsiella pneumoniae*	
46	*Salmonella enterica*	
7487	*Escherichia coli*	
Top Hits	**Supgroup**	
5979	*Escherichia coli* JJ1886	
192	*Escherichia coli* NA114	
110	*Escherichia coli* SE15	
Spiked urine Run 1		
MinION		
Hits	**Proteobacterial genomes**	
9	*Citrobacter freundii*	
13	*Klebsiella oxytoca*	
16	*Shigella dysenteriae*	
29	*Shigella boydii*	
45	*Shigella flexneri*	
60	*Salmonella enterica*	
74	*Shigella sonnei*	
224	*Klebsiella pneumoniae*	
6331	*Escherichia coli*	
Top Hits	**Supgroup**	
3741	*E. coli* APEC O78	
485	*E. coli* LY180	
449	*E. coli* K-12 substr. MC4100	
160	*E. coli* O104:H4	
Spiked urine Run 2		
MinION		
Hits	**Proteobacterial genomes**	
1	*Acinetobacter lwoffii*	
1	*Acinetobacter nosocomialis*	
1	*Aeromonas hydrophila*	
1	*Azospirillum brasilense*	
1	*Chromobacterium violaceum*	
1	*Dickeya dadantii*	
1	*Enterobacter aerogenes*	
1	*Escherichia* sp.	
1	*Hahella chejuensis*	
1	*Herminiimonas arsenicoxydans*	
1	*Kluyvera georgiana*	
1	*Laribacter hongkongensis*	
1	*Methylobacillus* sp.	
1	*Methylomonas aminofaciens*	
1	*Pantoea ananatis*	
1	*Polaromonas* sp.	
1	*Providencia rettgeri*	

MinION		
Hits	**Proteobacterial genomes**	
1	*Pseudomonas migulae*	
1	*Rhizobium etli*	
1	*Shewanella* sp.	
1	*Shuttle vector*	
1	*Xanthomonas euvesicatoria*	
1	*uncultured Shigella*	
2	*Achromobacter xylosoxidans*	
2	*Aeromonas caviae*	
2	*Enterobacteria phage*	
2	*Erwinia amylovora*	
2	*Photobacterium damselae*	
2	*Raoultella planticola*	
2	*Serratia liquefaciens*	
2	*Shigella* sp.	
2	*Vibrio nigripulchritudo*	
2	*Yersinia pestis*	
3	*Enterobacter asburiae*	
3	*Enterobacter hormaechei*	
3	*Yersinia enterocolitica*	
4	*Enterobacter cloa*	
4	*Vibrio cholerae*	
5	*Escherichia fergusonii*	
5	*Stenotrophomonas maltophilia*	
7	*Citrobacter freundii*	
7	*Delftia acidovorans*	
8	*Citrobacter rodentium*	
8	*Pseudomonas aeruginosa*	
9	*Haemophilus influenzae*	
11	*Acinetobacter baumannii*	
12	*Delftia* sp.	
12	*Serratia marcescens*	
15	*Cronobacter sakazakii*	
19	*Klebsiella oxytoca*	
126	*Shigella dysenteriae*	
190	*Pseudomonas putida*	
191	*Salmonella enterica*	
421	*Klebsiella pneumoniae*	
852	*Shigella flexneri*	
1418	*Shigella boydii*	
1925	*Shigella sonnei*	
11538	*Escherichia coli*	
Top Hits	**Supgroup**	
6618	*Escherichia coli* str. K12 substr. W3110 DNA,	
6506	*Escherichia coli* APEC O78	