Diversity of sequence types, plasmids and extended-spectrum β-lactamase genes among *Escherichia coli* from humans, animals and food in Germany, Netherlands and United Kingdom

Michaela J. Day[1*], Irene Rodríguez[2,3], Alieda van Essen-Zandbergen[4], Cindy Dierikx[4], Kristina Kadlec[5], Anne-Kathrin Schink[5], Guanghui Wu[6], Marie A. Chattaway[1], Vivienne DoNascimento[1], John Wain[7], Reiner Helmuth[2], Beatriz Guerra[2], Stefan Schwarz[5], John Threlfall[1], Martin J. Woodward[8], Nick Coldham[6], Dik Mevius[4,9] and Neil Woodford[1]

[1] Public Health England, London, United Kingdom, [2] Federal Institute for Risk Assessment (BfR), Berlin, Germany, [3] University Hospital Ramón y Cajal, and Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Madrid, Spain, [4] Central Veterinary Institute (CVI) of Wageningen UR, Lelystad, The Netherlands, [5] Institute of Farm Animal Genetics, Friedrich-Loeffler-Institut (FLI), Neustadt-Mariensee, Germany, [6] Animal and Plant Health Agency (APHA, Weybridge), Addlestone, United Kingdom, [7] University of East Anglia, Norwich, United Kingdom, [8] The University of Reading, Whiteknights, Reading, United Kingdom, [9] Utrecht University, Utrecht, The Netherlands.

Running title: *E. coli* diversity in animals, humans and food in Western Europe

Keywords: MLST, antimicrobial resistance, plasmids, CTX-M

***Corresponding author:** Dr Michaela J. Day, Antimicrobial Resistance and Healthcare Associated Infections (AMRHAI) Reference Unit, Public Health England, Reference Microbiology Services, 61 Colindale Avenue, London, NW9 5EQ, UK

Synopsis

Objectives: This study aimed to compare ESBL-producing *Escherichia coli* causing infections in humans with infecting or commensal isolates from animals and isolates from food of animal origin in terms of the strain types, the ESBL gene present and the plasmids that carry the respective ESBL genes.

Methods: A collection of 353 ESBL-positive *E. coli* isolates from the UK, The Netherlands and Germany was studied by MLST, ESBL gene identification and characterization of ESBL gene-carrying plasmids by PCR-based replicon typing (PBRT). Moreover, IncI1-Iγ and IncN plasmids were characterized by plasmid multilocus sequence typing (pMLST).

Results: The ESBL-producing *E. coli* represented 158 different STs with ST131, ST10 and ST88 being the most common. Overall, $bla_{CTX\text{-}M\text{-}1}$ was the most frequently detected ESBL gene, followed by $bla_{CTX\text{-}M\text{-}15}$, which was the most common ESBL gene in the human isolates. The most common plasmid replicon type overall was IncI1-Iγ followed by multiple IncF replicons.

Conclusions: ESBL genes were present in a wide variety of *E. coli* STs. IncI1-Iγ plasmids that carried the $bla_{CTX\text{-}M\text{-}1}$ gene were widely disseminated amongst STs in isolates from animals and humans whereas other plasmids and STs appeared to be more restricted to isolates from specific hosts.

Introduction

Extended-spectrum cephalosporin antibiotics have been used to treat a variety of infections caused by Gram-negative bacteria since the 1980s.[1] Resistance increased rapidly during the 2000s due to the dissemination of CTX-M-type enzymes, which are now the most prevalent ESBLs worldwide. ESBL-carrying *Escherichia coli* can be isolated from non-human sources, including companion and food-producing animals. The potential for ESBL-producing *E. coli* strains from animals to cause human infections has long been suggested, but as yet has not been firmly confirmed or refuted.[2] Certainly there is a diverse collection of ESBL genes and *E. coli* strains amongst animals globally.[3,4] There is potential for this reservoir to affect humans either by direct transmission of resistant strains or by them serving as donors for horizontal exchange of plasmids carrying ESBL genes.

This study aimed to investigate the extent of similarities between ESBL-producing *E. coli* causing infections in humans and those from animal and food sources in three European countries.

Materials and methods

Isolate selection

A total of 353 *E. coli* isolates with phenotypic resistance to ampicillin and cefotaxime (according to EUCAST guidelines)[5] were studied from the European ESBL-SAFEFOODERA project collection (EU ERA-Net, Ref. 08176).[6] The isolates were selected from national antimicrobial resistance surveillance programmes or from participants' routine diagnostic or reference laboratory activities and isolated between 2005-2009 (Table S1).

Plasmid and ESBL characterisation

ESBL and plasmid-mediated AmpC (pAmpC) genes had previously been identified to family level (e.g. TEM, SHV or CTX-M) and sub-family group (e.g. CTX-M group 1 or CTX-M group 9) level using a commercial microarray.[6] They were further characterised by PCR and DNA sequencing as described previously.[7]

Plasmid DNA was extracted from *E. coli* isolates and transfered into competent *E. coli* DH10B cells by electroporation (Life Technologies, Paisley, UK) as described.[8] Transformants that had acquired ESBL-encoding plasmids were selected on LB agar containing 1 mg/L cefotaxime. The replicon types of the ESBL-encoding plasmids were determined by PCR-based replicon typing (PBRT) as described,[9] except that the IncHI2 PCR was performed as a simplex reaction. Additional PCRs were used for IncR, IncU and ColE plasmids.[10] Plasmids belonging to replicon types IncI1-Iγ and IncN were further characterised using the plasmid multilocus sequence typing (pMLST) schemes and assigned to plasmid sequence types (pSTs) using the primers and conditions described on the PubMLST website (http://pubmlst.org/plasmid).

Molecular characterisation of E. coli strains

Genomic DNA was extracted from *E. coli* isolates using the Promega Wizard Kit (Promega, Southampton, UK). MLST was performed using the 'Achtman scheme' as previously described.[11] Data were analysed using BioNumerics (Applied Maths v.6.1) and figures generated using the minimum spanning tree for categorical data tool.

Results and discussion

Molecular characterisation of E. coli strains

MLST identified 158 different STs amongst the 353 ESBL-producing isolates. Fewer STs were found amongst the 134 ESBL-positive *E. coli* from humans than amongst the 219 isolates from animals and other sources (52 vs 125, respectively), although this apparent difference in diversity may have been biased by 'convenience sampling' of isolates in the collection, with no human commensal isolates included in the study. In humans, 44.0% (59/134) of the isolates belonged to the three most common human STs (ST131, ST405 and ST10/38, Table S2) compared with 34.9% (22/63) of the most common STs in cattle (ST88, ST69 and ST10) and only 19.7% (27/137) in poultry (ST10, ST88 and ST665). Overall, the ten most frequently identified STs (131, 10, 88, 405, 117, 58, 69, 38, 665, 156) (Table S2) accounted for 43.9% (155/353) isolates and eight of these were present among isolates from both animals and humans; the two exceptions were ST405 (humans only) and ST665 (poultry only). In total, 17 STs were shared by *E. coli* from both animals and humans.

Overall, ST131 was the most common ST identified (43 isolates; 12.2%) in this collection. The majority (40/43) of ST131 isolates were from humans, although three were from poultry samples (one each from retail meat and caecal contents from The Netherlands and one from faeces from Germany). The next most common STs were ST10 (26 isolates; 7.4%) followed by ST88 (23 isolates; 6.5%), both of which were isolated from a variety of sources in all three countries (Table S2). This result is supported by previous studies that have shown *E. coli* ST88 to be globally distributed in animals and humans[12,13] and to a further extent also ST10, [14-16] suggesting that there is potential that these STs could be circulating between animals and humans. However, more discriminatory typing via whole genome sequencing would be necessary to confirm this.[17] Other STs seemed to be more host-restricted, although low numbers of representatives in this collection of some STs prevented robust analysis. For example,

ST131, ST405 and ST38 appeared to be more frequently found in ESBL-producing isolates from humans (Table S2).

ESBL genes and replicon types of ESBL gene-carrying plasmids

Overall, *bla*$_{\text{CTX-M-1}}$ was the most common ESBL gene (132/353, 37.4%), followed by *bla*$_{\text{CTX-M-15}}$ (87/353, 24.6%) (Table 1). The *bla*$_{\text{CTX-M-1}}$ gene was the most frequent ESBL gene in poultry isolates (64/137, 46.7%) in all three countries and from cattle in Germany (22/26, 84.6%) (Table S3). This differs from cattle in the UK where *bla*$_{\text{CTX-M-15}}$ was most common (17/34, 50%) (Table S3). The pAmpC gene *bla*$_{\text{CMY-2}}$ was the second most frequent cefotaxime resistance gene in poultry isolates from Germany (4/18, 22.2%); *bla*$_{\text{TEM-52c}}$ in the Netherlands (18/86, 20.9%) and *bla*$_{\text{CTX-M-14}}$ in the UK (7/33, 21.2%) (Table S3). The *bla*$_{\text{CTX-M-15}}$ gene was common in human isolates in all three countries (67/134 50.0%), although in humans in the Netherlands (19/66, 28.8%) and Germany (5/14, 35.7%) *bla*$_{\text{CTX-M-1}}$ was far more frequent than in the UK (2/54, 3.7%) (Table S3).

Transformation of plasmids that conferred cefotaxime resistance into *E. coli* DH10B was successful for 341/353 (96.6%) donor isolates. Replicon typing of the transformed plasmids revealed that IncI1-Iγ plasmids were most common overall (142/341, 41.6%) (Table 1). The IncI1-Iγ plasmids were widely disseminated among isolates from different sources and countries although they were predominant in cattle from the UK and Germany and in humans and poultry from the Netherlands (Table S4).

Complexity of ST-ESBL gene-plasmid combinations

In this collection of 353 cefotaxime-resistant *E. coli* from three countries, we identified 158 STs, 16 different ESBL genes or the pAmpC gene *bla*$_{\text{CMY-2}}$ and 19 different plasmid replicon types among the 341 transferable plasmids carrying these genes. This complexity makes it difficult to seek direct evidence for possible overlap between human

and non-human reservoirs in this collection. Despite the diversity observed, the study identified a number of dominant *E. coli* ST-plasmid-ESBL gene combinations, some of which were found only in isolates from humans, such as ST131 and ST405 with multiple IncF $bla_{CTX\text{-}M\text{-}15}$–carrying plasmids or only in animals, such as ST88 with IncI1-Iγ $bla_{CTX\text{-}M\text{-}1}$–carrying plasmids. In contrast, other combinations were found in isolates from all sources, such as ST10, ST58 and ST117 with IncI1-Iγ plasmids.

Of 142 transformants carrying IncI1-Iγ plasmids 89 (62.7%) were positive for $bla_{CTX\text{-}M\text{-}1}$ (Table 2). The plasmid donor? isolates belonged to 56 different STs, with ST10 (ten isolates plus seven single (SLV) and one triple locus variants (TLV)) and ST88 (ten isolates plus three SLVs and two double locus variants (DLVs)) being the most common STs. Analysis of a subset of 128 IncI1-Iγ plasmids by pMLST revealed considerable diversity. The analysed IncI1-Iγ plasmids were assigned to 16 pMLST types of which pST7 (43/128, 33.6%) and pST3 (27/128, 21.1%) were the most common as previously described.[18] Plasmids displaying the IncN replicon type (22/341, 6.5%) were associated with human isolates, with most of them (21/22, 95.5%) harbouring $bla_{CTX\text{-}M\text{-}1}$. The pMLST of the IncN plasmids showed that all represented pST1. The IncK replicon type was frequent in poultry and meat isolates from all three countries (Table S4), most of these plasmids carried $bla_{CTX\text{-}M\text{-}14}$ (13/24, 54.2%).

Multiple IncF replicons were seen in 56/341 (16.4%) plasmids, and consisted of eight different *rep* combinations (Table S5). Five of these combinations were found exclusively in human isolates, the three others were found in both animal and human isolates. Plasmids that exhibited IncF alone were present in 10 isolates (10/341 2.9%) with half of these isolates originating from cattle faecal samples and carrying $bla_{CTX\text{-}M\text{-}14}$.

There is potential for plasmid/ESBL gene transmission between *E. coli* from humans, animals and food. IncI1-Iγ plasmids harbouring $bla_{CTX\text{-}M\text{-}1}$ were common in animal isolates and were also seen in human *E. coli* isolates of 56 different STs. Other

plasmids seemed to be less widely disseminated, such as the multiple IncF *bla*$_{CTX-M-15}$-bearing plasmids. These conclusions are supported by data from other national studies performed on more recent isolates in each of the countries.[19,20] In the future, it would be interesting to further investigate (i) the relatedness of *E. coli* isolates that shared the same STs by whole genome sequencing and (ii) the mechanisms by which certain plasmids are adaptable to multiple hosts/*E. coli* types while others are seemingly host restricted.

Acknowledgements

We thank Dr. E. Tietze (Robert Koch Institute, Wernigerode, Germany) for the German human isolates and Dr. Maurine Leverstein–van Hall (University Medical Center of Utrecht, The Netherlands, on behalf of the national ESBL surveillance group) for the Dutch human isolates.

Funding

This work was supported by the EU-SAFEFOODERA project EU ERA-Net, Ref. 08176, entitled 'The role of commensal microflora of animals in the transmission of extended spectrum β-lactamases'. The UK based work was funded by the Food Standards Agency. The contribution of KK, A-KS, and SS was supported by internal financial support of the FLI. During the experimental execution of this work, IR was a postdoctoral student at the BfR (Berlin, Germany), with a grant from the Fundación Ramón Areces (Madrid, Spain); she currently holds a postdoctoral position associated with a Sara Borrell contract (Ref. CD12/00492; Instituto de Salud Carlos III, Spain) at University Hospital Ramón y Cajal (Madrid, Spain).

Transparency declarations

NW has no personal interests to declare. However, Public Health England's Antimicrobial Resistance and Healthcare Associated Infections Reference Unit has received financial support from numerous sources, including: AchaogenInc, AllecraAntiinfectives GmbH, Amplex, AstraZeneca UK Ltd, Becton Dickinson Diagnostics, bioMerieux, Bio-Rad Laboratories Ltd., The British Society for Antimicrobial Chemotherapy (BSAC), Cepheid, Check-Points B.V, Cubist Pharmaceuticals, Department of Health, Enigma Diagnostics Ltd., Food Standards Agency, Glaxo SmithKline Services Ltd, Henry Stewart Talks, IHMA Ltd, Merck Sharpe & Dohme Corp, Meiji Seika Kiasya Ltd, MelintaTherapeutics Inc., Momentum Bioscience Ltd., Nordic Pharma Ltd, Norgine Pharmaceuticals, Rempex Pharmaceuticals Ltd, Rokitan Ltd, Smith & Nephew UK Ltd, Tetraphase Pharmaceuticals, Trius Therapeutics, VenatoRx, Wockhardt Ltd.

Other authors: None to declare

References

1. Paterson DL, Bonomo RA. Extended-spectrum β-lactamases: a clinical update. *Clin Microbiol Rev* 2005; **18**: 657-86.

2. Liebana E, Carattoli A, Coque TM, *et al*. Public health risks of enterobacterial isolates producing extended-spectrum β-lactamases or AmpC β-lactamases in food and food-producing animals: an EU perspective of epidemiology, analytical methods, risk factors, and control options.*Clin Infect Dis* 2013; **56**: 1030-7.

3. Briñas L, Moreno MA, Teshager T, *et al*. β-Lactamase characterization in *Escherichia coli* isolates with diminished susceptibility or resistance to extended-spectrum cephalosporins recovered from sick animals in Spain. *Microb Drug Resist* 2003; **9**: 201-9.

4. Fischer J, Rodríguez I, Baumann B, *et al*. *bla*$_{\text{CTX-M-15}}$-carrying *Escherichia coli* and *Salmonella* isolates from livestock and food in Germany. *J Antimicrob Chemother* 2014; **69**: 2951-8.

5. EUCAST clinical breakpoint guidelines table version 5, http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_5.0_Breakpoint_Table_01.pdf

6. Wu G, Day MJ, Mafura MT, *et al*. Comparative analysis of ESBL-positive *Escherichia coli* isolates from animals and humans from the UK, The Netherlands and Germany. *PLoS One* 2013; **8**: e75392.

7. Rodríguez I, Barownick W, Helmuth R, *et al*. Extended-spectrum β-lactamases and AmpC β-lactamases in ceftiofur-resistant *Salmonella enterica* isolates from food and livestock obtained in Germany during 2003-07. *J Antimicrob Chemother* 2009; **64**: 301-9.

8. Rodríguez I, Thomas K, van Essen A, *et al*. Chromosomal location of *bla*$_{CTX-M}$ genes in clinical isolates of *Escherichia coli* from Germany, The Netherlands and The United Kingdom. *Int J Antimicrob Agents* 2014; **43**: 553-7.

9. Carattoli A, Bertini A, Villa L, *et al.* Identification of plasmids by PCR-based replicon typing.*J Microbiol Meth;* 2005; **63**: 219-28.

10. García-Fernández A, Fortini D, Veldman K, *et al.* Characterization of plasmids harbouring *qnrS1*, *qnrB2* and *qnrB19* genes in *Salmonella*. *J Antimicrob Chemother* 2009; **63**: 274-81.

11. Wirth T, Falush D, Lan R, *et al.* Sex and virulence in *Escherichia coli*: an evolutionary perspective. *Molecular Microbiology* 2006; **60**: 1136–51.

12. Timofte D, Maciuca IE, Evans NJ, *et al.* Detection and molecular characterization of *Escherichia coli* CTX-M-15 and *Klebsiella pneumoniae* SHV-12 β-lactamases from bovine mastitis isolates in the United Kingdom. *Antimicrob Agents Chemother.* 2014; **58**: 789-94.

13. Guillouzouic A, Caroff N, Dauvergne S, *et al.* MLST typing of *Escherichia coli* isolates overproducing AmpC β-lactamase. *J Antimicrob Chemother* 2009; **63**: 1290-2.

14. Overdevest I, Willemsen I, Rijnsburger M, *et al.* Extended-spectrum β-lactamase genes of *Escherichia coli* in chicken meat and humans, The Netherlands. *Emerg Infect Dis* 2011; **17**: 1216-22.

15. Cohen Stuart J, van den Munckhof T, Voets G, *et al.* Comparison of ESBL contamination in organic and conventional retail chicken meat. *Int J Food Microbiol* 2012; **154**: 212-4.

16. Ewers C, Bethe A, Semmler T, *et al.* Extended-spectrum β-lactamase-producing and AmpC-producing *Escherichia coli* from livestock and companion animals, and their putative impact on public health: a global perspective. *Clin Microbiol Infect* 2012; **18**: 646-55.

17. de Been M, Lanza VF, de Toro M, et al. Dissemination of cephalosporin resistance genes between Escherichia coli strains from farm animals and humans by specific plasmid lineages. *PLoS Genet*. 2014; **18**:10(12)

18. Smith H, Bossers A, Harders F, *et al.* Characterization of epidemic IncI1-Iγplasmids harboringAmbler class A and C genes in *Escherichia coli* and *Salmonella enterica* from animals and humans. *Antimicrob Agents Chemother* 2015; **59**: 5357-65.

19. Valentin L, Sharp H, Hille K, *et al.* Subgrouping of ESBL-producing *Escherichia coli* from animal and human sources: an approach to quantify the distribution of ESBL types between different reservoirs. *Int J Med Microbiol* 2014; **304**: 805-16.

20. Leverstein-van Hall MA, Dierikx CM, Cohen Stuart J, *et al.* Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains. *Clin Microbiol Infect* 2011; **17**: 873-80.

Table 1: **Associations between major *E. coli* sequence types, ESBL genes and ESBL-encoding plasmids**

		ESBL/pAmpC																	Replicon type							
		CTX-M-group-1					CTX-M-group-9						SHV			TEM		pAmpC								
ST	ESBL/pAmpC identified (%)[c]	CTX-M-1	CTX-M-3	CTX-M-15	CTX-M-32	CTX-M-51	CTX-M-55	CTX-M-9[b]	CTX-M-14	CTX-M-14B	CTX-M-27	CTX-M-2	SHV-2	SHV-12	TEM-19	TEM-52	TEM-52c	CMY-2	IncA/C	IncF	>1 IncF	IncI1-Iγ	IncK	IncN	Other [a]	No Transformants
131	43 (100)	1		34					2		1	1		1			2	1		1	28	6	1		5	2
10	25 (96)	10	1	4	1		1		1	3		1				1	2			2	3	12	1	2	6	
88	15 (65)	12		2													1					10		1	11	1
405	12 (100)			12																	9	2			1	
117	10 (100)	6		1					1	1							1			1		6			3	
58	9 (100)	3	2	2					1								1		1			7	1			
69	9 (100)	8		1																		4		1	4	
38	8 (100)	1		3		1			3													1			5	2
665	7 (88)	1		1								1					2	2	2		1	3			2	
156	4 (57)	1		1								1					1					2			4	1
Other	181 (91)	89	1	26			2	3	17			3	1	5	1	2	18	13	2	6	15	89	21	18	41	6
TOTAL	**323 (92)**	**132**	**4**	**87**	**1**	**1**	**3**	**3**	**25**	**4**	**1**	**7**	**1**	**6**	**1**	**3**	**28**	**16**	**5**	**10**	**56**	**142**	**24**	**22**	**82**	**12**

[a] Other replicon types Includes; 6 IncHI2, 7 IncB/O, 1 ColE, 1 IncFIA, 3 IncHI2 and? IncP, 2 IncB/O and? IncP and 62 non-identifiable

[b] Includes one CTX-M-9 with point mutations at nucleotide 93 (G →T) and 701 (T→C) which are both synonymous mutations

[c] 30 isolates had phenotypic resistance to ampicillin and cefotaxime but the gene responsible was not identified

Table 2: Association of ESBL genes with plasmid replicon types

		ESBL/pAmpC																		
		CTX-M-group-1						CTX-M-group-9					SHV		TEM			pAmpC	
Rep type	Number	CTX-M-1	CTX-M-3	CTX-M-15	CTX-M-32	CTX-M-51	CTX-M-55	CTX-M-9	CTX-M-14	CTX-M-14B	CTX-M-27	CTX-M-2	SHV-2	SHV-12	TEM-19	TEM-52	TEM-52c	CMY-2	unknown
IncI1-lγ	142	89	3	22										2			24	2	
>1 IncF	56	5		45			1		2		1			1					1
IncK	24								13			1						9	1
IncN	22	21							1										
Frep	10			2			2		5	1									
IncA/C	5		1														1	3	
Other[a]	82	17		15	1			2	3	3		5		3	1	3	3	1	25
No transformants obtained	12			3		1		1	1			1	1					1	3
Grand Total	353	132	4	87	1	1	3	3	25	4	1	7	1	6	1	3	28	16	30

[a] Other replicon types Includes; 6 IncHI2, 7 IncB/O, 1 ColE, 1 IncFIA, 3 IncHI2 and? IncP, 2 IncB/O and IncP and 62 non-identifiable