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Detection of *Cryptosporidium hominis* by clinical metagenomics in stool samples from an outbreak of diarrhoea among British military personnel in Kenya

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ABSTRACT

Introduction Traveller's diarrhoea is a common complaint among deployed military personnel. Maintaining sample integrity prior to diagnostic testing is a key challenge in resource-limited environments. We report the comparison of three long-term ambient temperature stool sample stabilisation matrices for the detection of *Cryptosporidium hominis* from samples collected during an outbreak among British military personnel stationed in Kenya.

Methods A retrospective cohort of stool samples, each stabilised for more than 12 months at ambient temperatures using Flinders Technical Associate (FTA) cards, OMNIgene GUT tubes and DNA Shield faecal collection tubes, were analysed by Nanopore-based clinical metagenomic (CMgs) DNA sequencing and quantitative real-time PCR (qPCR) in the UK. The results were compared with BioFire FilmArray Gastrointestinal Panel testing carried out at the point of sampling in Kenya.

Results *Cryptosporidium* DNA was detected in 13/24 (54.2%) OMNIgene GUT samples by CMg following long-term storage, compared with 9/24 (37.5%) of DNA Shield samples. Samples stored on FTA cards did not identify *Cryptosporidium* DNA by CMg in any sample. OMNIgene GUT samples also had the highest rate of detection of *C. hominis* DNA by qPCR, with 23/24 samples testing positive, compared with 21/24 and 17/20 of DNA Shield and FTA samples, respectively.

Conclusions Samples stored in OMNIgene GUT tubes retained detectable levels of *Cryptosporidium* DNA in a higher proportion of samples following long-term storage. This study demonstrates the importance of selecting the optimal sample collection and stabilisation matrix for CMg and qPCR based diagnostic testing in austere environments.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Clinical metagenomics (CMgs) represent a promising future technology for the untargeted detection of enteric pathogens in deployed military healthcare facilities. Selection of the optimal stabilisation matrix is key to the successful detection of enteric pathogens.

WHAT THIS STUDY ADDS

⇒ *Cryptosporidium* DNA was detected at a higher rate following long-term storage from samples stored in OMNIGene GUT tubes, compared with DNA Shield and Flinders Technical Associate cards.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study will help to inform the future development of CMg methods and interpretation of CMg outputs, with the aim of improving deployed diagnostics. Further evaluation of OMNIGene GUT tubes for the sampling and long-term storage of stool samples is recommended.

INTRODUCTION

Traveller's diarrhoea (TD) is defined as the passage of three or more loose stools within a 24-hour period, alongside additional symptoms including vomiting, abdominal cramps, nausea, fever, blood or mucus in the stools and faecal urgency, experienced while travelling or within 10 days of travel.[1] TD is usually the most common medical complaint among deployed military personnel, resulting in reduced performance, lost ability to work and in some cases additional gastrointestinal (GI) disorders, increasing pressures on the provision of deployed healthcare.[2 3]

The majority of cases of TD in military populations are linked to bacterial pathogens, varying according to region.[4] Species belonging to the *Cryptosporidium* genus are protozoan enteric parasites with a global distribution and, while less common than bacterial pathogens, are a significant cause of TD with limited effective therapeutic options.[5] Two species, *C. hominis* and *C. parvum* are responsible for 95% of infections in humans, with *C. hominis* being the most common.[6] Transmission is via the faecal-oral route through contaminated water, food or direct contact with humans or animals.[7]

In deployed military environments, the aetiology of TD can be determined using PCR based tests, including the multiplex FilmArray (BioFire) GI Panel (BioMerieux), targeting 22 common enteric pathogens, including viruses, bacteria and *Cryptosporidium*, among other parasites.[8] This platform provides highly accurate detection of *Cryptosporidium* spp., with previous evaluations demonstrating 100% sensitivity and a specificity

of ≥97.1%.[9] Microscopic identification of parasites can also be carried out, however, this method achieves moderate sensitivities of 70%–80% and has a high operative and logistical burden unsuited for some deployed environments.[10]

Clinical metagenomics (CMgs) is defined as the untargeted sequencing of the genetic content of a sample without the prior isolation of an organism,[11] and has the potential to provide an additional diagnostic test. Recent developments in DNA sequencing technology include nanopore-based sequencing platforms that use small, portable equipment and simple sample preparation methods to generate sequencing data suitable for real-time analysis. This, combined with advances in sample preparation technologies, including DNA extraction and DNA sequencing library preparation, has progressed CMg towards being suitable for use in austere environments such as deployed medical treatment facilities. However, the collection and stabilisation of stool samples is a key challenge for CMg, particularly where immediate testing is not available or the repatriation of samples to a separate diagnostic laboratory is required.

In February 2022, there was an outbreak of TD among British Army personnel stationed in Kenya, caused by *C. hominis*.[12] In this study we compare the performance of three commercially available stabilisation matrices for the detection of *Cryptosporidium* by CMg and quantitative real-time PCR (qPCR) following long-term storage from a retrospective cohort of stool samples collected during this TD outbreak event.

METHODS

Sample collection

Following an increase in reports of profuse watery diarrhoea among British Army personnel stationed in Nanyuki, Kenya, an outbreak investigation was conducted. This included on-site testing of stool samples using the FilmArray (BioFire) GI Panel followed by molecular characterisation of repatriated samples by the *Cryptosporidium* Reference Unit (CRU; Swansea, UK). This investigation identified a rare *C. hominis* subtype as the cause of the outbreak.[12] Other bacterial and viral enteropathogens were also detected. Samples were prepared in Kenya for long-term storage using Flinders Technical Associate (FTA cards (Whatman), OMNIgene GUT tubes (DNAgenotek) and DNA Shield Faecal Collection Tube (Zymo Research) stabilisation matrices.

Samples were stored at ambient temperature for >12 months using three stabilisation matrices[13] and retrospectively selected for CMg analysis; OMNIgene GUT tubes (n=39), DNA Shield Faecal Collection Tubes (n=39) and FTA cards (n=35). Four *Cryptosporidium* positive samples were not stored on FTA cards and were unavailable for CMg analysis.

DNA extraction

The DNA was extracted from 250 µL of the OMNIgene GUT and DNA Shield samples using the QIAmp PowerFaecal Pro DNA extraction kit (Qiagen) according to the manufacturer's instructions. The bead beating was carried out in Matrix Lysing E tubes (MP Biomedicals) for 60 s at 6.0 m/s on a FastPrep-24 5G (MP Biomedicals) and the DNA was eluted into 50 µL of Solution C6. The DNA was eluted directly from the FTA cards according to a previously described method[14] and was eluted into 50 µL nuclease-free water. Negative controls consisting of 250 µL phosphate buffer saline (PBS) only and positive controls consisting of 30 µL microbial community standard II (log distribution) (Zymo Research) and 220 µL PBS were processed alongside each batch of samples.

DNA quantification

The total DNA concentration was quantified using the double-stranded DNA high-sensitivity assay kit on the Qubit 4 fluorimeter (Invitrogen) according to the manufacturer's instructions.

DNA purification

The 50 µL DNA extract was purified by AMPure XP bead purification according to the manufacturer's instructions at a ratio of 1.8× volume of beads to DNA extract in a 1.5 mL LoBind tube and eluted into 10 µL nuclease-free water.

Rapid PCR barcoding library preparation method

The DNA was prepared for sequencing using the Rapid PCR Barcoding library preparation kit V14 (SQK-RPB114.24) (Oxford Nanopore Technologies) (RPCR) according to the manufacturer's instructions without amendment. An R10.4.1 flow cell (Oxford Nanopore Technologies) was then primed for sequencing according to the manufacturer's instructions and the libraries loaded onto the flow cell. Prepared libraries were sequenced for 24 hours and the data stored in POD5 format.

Bioinformatic analysis

The POD5 files were base-called and Phred quality scores (Qscores) calculated with Guppy software V.6.2.x. A previously developed bioinformatics pipeline for the taxonomic profiling of microbial species, including *Cryptosporidium* spp., was applied.[15] Human reads were removed by mapping against the *Homo sapiens* reference genome CHM13v2 using minimap2 (V.2.25). Unmapped reads were included for mapping against the RefSeq database using minimap2 (V.2.25) and screening AMR genes using KMA (V.1.4.9) tool with the amrfinderplus database (V.3.11.4).

Microbial species were considered present if ≥5 reads, minus the number of reads of the same species identified in the negative control sample, matched that species. Data were analysed using GraphPad Prism 10 (V.10.0.2) software. p-values were obtained by performing a one-way ANOVA or mixed-effects analysis with a post hoc Tukey's test.

Principal coordinate analysis (PCoA) of the β-diversity Bray-Curtis distance was calculated using the *pcoa* and *vegdist* functions within the *ecodist* R package.[16] Species α-diversity was calculated using the Shannon diversity index.[17]

Real-time PCR

A published *C. hominis Lib13* gene Taqman probe based qPCR assay[18] was performed in triplicate on all samples using the QuantStudio 7 Flex (ThermoFisher) platform and quantitation cycle (Cq) values determined by the accompanying software. Each run included synthetic *C. hominis* DNA as a positive control (PRA-3011SD, ATCC) and nuclease-free water as a negative control. Reactions used TaqMan Fast Advanced Master Mix (Applied Biosystems) in a 25 µL total volume containing 500 nM forward and reverse primers, 250 nM probe and 2.0 µL template DNA. Thermal cycling comprised 2 min at 95 °C followed by 45 cycles of 10 seconds at 95 °C and 1 min at 60 °C.

Ethics statement

Ethical approval for the collection and additional testing of the primary stool samples was provided by the Ministry of Defence Research Ethics Committee (MODREC) (2076/MODREC/21). All samples were anonymised prior to transfer to the Defence Science and Technology Laboratory (Dstl) for CMg analysis.

Table 1 Detection of *Cryptosporidium* by CMg from known positive stool samples following long-term, ambient temperature storage in DNA Shield, OMNIgene GUT and FTA cards

	DNA shield (n=24)	OMNIgene GUT (n=24)	FTA (n=21)
Mean number of *Cryptosporidium* reads per sample (min–max)	52.13 (0–324)	37.88 (0–339)	0.48 (0–4)
Mean *Cryptosporidium* read length per sample (bp) (min–max)	3545 (2081–4680)	3757 (3022–4948)	3614 (2150–5103)
Percentage of known positive samples that detected *Cryptosporidium* by CMg	37.50%	54.17%	0.00%

Values are the mean of the data. DNA Shield and OMNIgene GUT n=24. FTA n=21.
CMg, clinical metagenomic; FTA, Flinders Technical Associate.

RESULTS

Detection of *Cryptosporidium*

Study population

Of the 1200 personnel stationed at Nanyuki at the time of the outbreak, 172 (14.3%) individuals presented with symptoms of TD, with 106 (61.6%) individuals providing faecal samples for FilmArray GI panel testing.

A retrospective cohort of these stool samples was processed for CMg following repatriation to the UK and long-term storage. This included sixteen (41.0%) samples positive for *Cryptosporidium* only, eight (20.5%) for *Cryptosporidium* plus additional pathogen(s), eight positive for bacterial pathogens only, two for non-*Cryptosporidium* parasites and one was polymicrobial; four samples (10.3%) tested negative.

Clinical metagenomics

Applying the predefined thresholds, the OMNIgene GUT samples achieved the highest rate of *Cryptosporidium* detection by CMg, in 13 of the 24 samples (54.2%) that tested positive by FilmArray GI Panel at the point of sampling. This was higher than the DNA Shield samples at 9/24 (37.5%) and the FTA samples, with no detection of *Cryptosporidium* in any known positive samples. There were no false-positive detections of *Cryptosporidium* in any negative sample (table 1).

qPCR

The OMNIgene GUT samples also detected *C. hominis* DNA by qPCR in 23/24 positive samples, compared with 21/24 DNA Shield samples. Despite not being detected by CMg, *C. hominis* DNA was detected by qPCR in 17/20 FTA positive samples. OMNIgene GUT samples also had an observed higher concentration of *Cryptosporidium* DNA across all positive samples, as represented by a decreased mean Cq value of 33.5, compared with the DNA Shield and FTA samples with mean Cq values of 35.4 and 36.6, respectively (figure 1). There was one false-positive identification of *C. hominis* by qPCR in an FTA sample that had previously tested negative by FilmArray GI Panel at the point of sampling, identified at a Cq of 40.3. The *C. hominis* Cq values obtained from each sample are reported with the number of *Cryptosporidium* reads identified by CMg in online supplemental table S1.

Detection of additional pathogens

The detection of non-*Cryptosporidium* pathogens identified by the FilmArray GI Panel at the species and genus level was evaluated. The delineation of *Escherichia coli* pathotypes was not evaluated, due to the challenges associated with discriminating between each pathotype. The current CMg workflow was not developed to include the detection of RNA based viruses, and detection of astrovirus and norovirus was not evaluated. The comparison included the detection of *Clostridioides difficile*, *Campylobacter jejuni*, *Campylobacter coli*, *Campylobacter upsaliensis*, *Salmonella*, *Giardia duodenalis* and *Entamoeba histolytica*. There was no difference in detection between the DNA Shield and OMNIgene GUT samples, with CMg matching six of the ten FilmArray GI Panel results, compared with the FTA samples that identified five. There were no reads assigned to the enteric parasites *G. duodenalis* and *E. histolytica* in any sample.

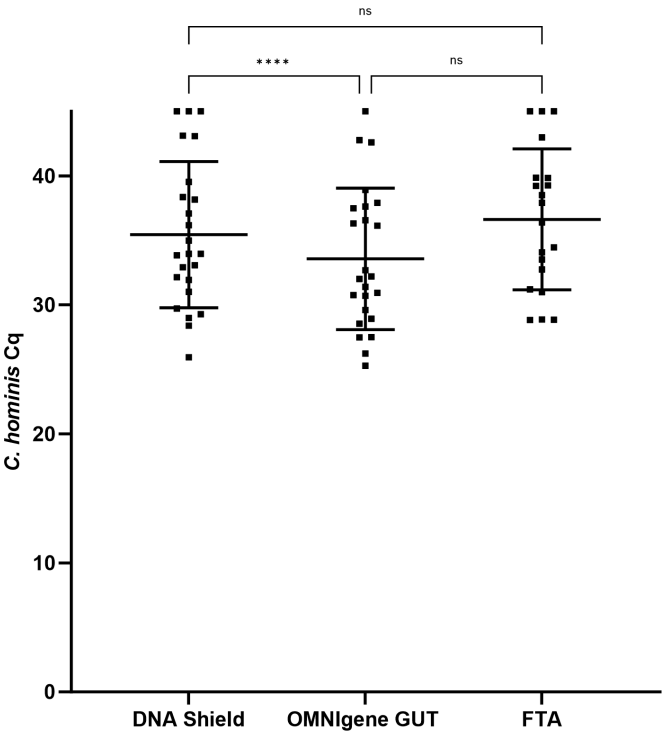

Figure 1 *Cryptosporidium hominis* quantitation cycle (Cq) values from known positive samples stored in three stabilisation matrices. DNA extracts were analysed by quantitative real-time PCR (qPCR) for the detection of *C. hominis* species-specific DNA. Cq values from samples stored in each storage method were compared. There was a significant reduction in Cq values obtained from OMNIgene GUT samples compared with the DNA Shield samples. Error bars represent the SD. The inner line represents the mean. DNA Shield n=24. OMNIgene GUT n=24. FTA n=20. ns p>0.05. ****p<0.0001. FTA, Flinders Technical Associate.

Classified microbial reads are sequencing reads assigned to known taxa by the bioinformatics pipeline, whereas unclassified reads lack sufficient similarity to reference genomes for taxonomic assignment. The proportion of classified reads assigned to a single species can be used to validate detection, as higher proportions increase confidence in true species identification. The number of and proportion of total classified microbial reads assigned to additional enteric pathogen species are reported in table 2.

Microbiome stabilisation

To evaluate the impact of the stabilisation matrix on the composition of microbial communities following long-term storage, the proportion of reads assigned to the ten most abundant phyla were compared (figure 2A). There was no significant alteration

Table 2 Detection of additional enteric pathogens by CMg

Sample	FilmArray GI panel positive (as per report sheet)	Total reads assigned to species/genus by CMg (proportion of classified microbial reads)		
		DNA shield	OMNIgene GUT	FTA
C1	*C. difficile*	142 (0.11)	161 (0.14)	70 (0.26)
B3	*C. difficile*	269 (0.18)	342 (0.21)	0 (0.00)
B40	*C. upsaliensis*	0 (0.00)	0 (0.00)	0 (0.00)
	C. coli	39 (0.12)	25 (0.10)	86 (0.65)
	C. jejuni	1276 (3.83)	698 (2.76)	993 (7.47)
B44	*C. upsaliensis*	0 (0.00)	0 (0.00)	0 (0.00)
	C. coli	6 (0.01)	9 (0.01)	3 (0.02)
	C. jejuni	221 (0.21)	303 (0.37)	69 (0.37)
B94	*G. duodenalis*	0 (0.00)	0 (0.00)	0 (0.00)
B020	*Salmonella*	1 (0.00)	0 (0.00)	0 (0.00)
B045	*C. upsaliensis*	0 (0.00)	0 (0.00)	0 (0.00)
	C. coli	183 (0.40)	343 (0.69)	228 (1.16)
	C. jejuni	5992 (13.22)	10 773 (21.70)	3932 (20.09)
B049	*C. upsaliensis*	0 (0.00)	0 (0.00)	0 (0.00)
	C. coli	0 (0.00)	0 (0.00)	0 (0.00)
	C. jejuni	2 (0.03)	1 (0.01)	2 (0.06)
B057	*C. upsaliensis*	0 (0.00)	0 (0.00)	0 (0.00)
	C. coli	11 (0.00)	9 (0.00)	40 (0.02)
	C. jejuni	432 (0.13)	245 (0.12)	552 (0.31)
B085	*E. histolytica*	0 (0.00)	0 (0.00)	0 (0.00)

The number and proportion of classified microbial reads assigned to the enteric pathogens identified by the FilmArray GI Panel from the primary stool samples from each stabilisation matrix are reported.
C. coli, *Campylobacter coli*; *C. difficile*, *Clostridiodes difficile*; *C. jejuni*, *Campylobacter jejuni*; CMg, clinical metagenomic; *C. upsaliensis*, *Campylobacter upsaliensis*; *E. coli*, *Escherichia coli*; FTA, Flinders Technical Associate; *G. duodenalis*, *Giardia duodenalis*.

to the average proportions of classified reads assigned to these phyla between any stabilisation matrices across all samples.

The Bray-Curtis distances were compared at the phylum (figure 2B) and species (figure 2C) level using PCoA. At the phylum level, the first and second principal components explained 65.3% of the differences in microbial composition and there was no significant difference between the stabilisation matrices ($p=0.86$). However, at the species level, there was a significant difference, with the first and second principal components explaining 29.5% of the differences in microbial composition ($p=0.001$) (figure 2C). When the α-diversity was compared at the species level, the FTA samples contained significantly lower diversity, while there was no significant difference between the OMNIgene GUT and DNA Shield samples (figure 2D).

DISCUSSION

CMg has the potential to be used as an untargeted diagnostic test for TD to complement methods currently available in deployed military treatment facilities. This study aimed to inform the future optimisation of CMg methods for the diagnosis of TD by comparing three long-term stool sample stabilisation matrices, DNA Shield, OMNIgene GUT and FTA cards, previously identified as effective stabilisation matrices,[19–21] for the detection of a protozoan enteric pathogen, *C. hominis*. *Cryptosporidium* DNA was detected at the highest rate by CMg and qPCR in samples stored in OMNIgene GUT tubes, compared with DNA Shield and FTA card samples. However, no differences were observed in the detection of additional enteric pathogens by CMg. FTA samples also showed the lowest species diversity compared with OMNIgene GUT and DNA Shield samples.

Samples stored using OMNIgene GUT achieved the highest rate of *Cryptosporidium* detection, despite the samples being stored beyond the manufacturer's recommended period[22] and DNA Shield and FTA cards being processed within their recommended storage periods.[23 24] In comparison, FTA cards failed to identify *Cryptosporidium* in any known positive sample by CMg. This indicates that OMNIgene GUT would be a suitable stabilisation matrix for further evaluation, working towards developing a deployed CMg diagnostic capability for enteric infections. However, *Cryptosporidium* spp. oocyst walls consist of a protein-lipid-carbohydrate matrix, conferring strong environmental resistance.[25] This may have enabled better preservation of oocysts during long-term storage than bacterial or viral pathogens and further evidence is required before OMNIgene GUT tubes can be recommended for long-term CMg detection of all enteric pathogens.

In a separate study, faecal samples from the same cohort were retested with the FilmArray GI Panel after storage in the three matrices[13] yielding similar sensitivities and specificities for *Cryptosporidium* spp. detection. Percentage Observed Agreement (POA) compared with fresh samples was 98.6% for OMNIgene GUT, 96.7% for DNA Shield and 71.9% for FTA cards. Tests were also performed for the four next most common bacterial pathogens identified in the field study[12 13]: enteroaggregative, enteropathic and *Shiga*-like toxin-producing *E. coli* and *Campylobacter* spp. Samples stored in OMNIgene GUT or DNA Shield showed POA≥89.8% across all tests, whereas FTA-stored samples demonstrated only moderate POA (67.2%–77.6%) compared with fresh testing.[13] These findings reinforce the need to validate faecal storage systems according to the investigation type and target enteropathogen.

Despite poor *Cryptosporidium* detection in FTA samples by CMg, no significant increase in the *C. hominis* Cq values was observed compared with the DNA Shield and OMNIgene GUT samples. FTA cards contain a patented chemical mixture to preserve DNA for downstream analysis.[26] It may be that card components were coeluted and inhibited DNA preparation for sequencing. Previous studies have also shown significant degradation of viral RNA during storage on FTA cards, particularly at elevated temperatures.[27] Increased *Cryptosporidium* DNA degradation could have reduced long-read DNA sequencing yield without impacting qPCR Cq values, which rely on shorter DNA fragments (<100 bp).

While OMNIgene GUT achieved the highest *Cryptosporidium* detection rate, the proportion of samples positive by CMg, using a threshold of five reads assigned to a species, remained low. Consequently, the previously identified *C. hominis* ImA13G1 subtype[12] could not be resolved, with reads incorrectly assigned to *C. parvum*. The austere field conditions meant primary samples were not stored at −80°C or processed immediately for CMg, limiting interpretation of the poor detection following long-term storage. Future studies should include analysis of primary samples stored at −80°C and processed immediately after collection to determine whether reduced detection reflects storage-related loss or low initial parasite burden. Semitargeted enrichment steps[28] may further improve detection of low-abundance enteric pathogens.

The threshold of five reads assigned to a species to confirm a positive CMg identification was arbitrarily selected based on previous nanopore-based CMg studies.[15] Selecting an appropriate threshold for detecting low abundance species within the complex gut microbiome is challenging. A high threshold

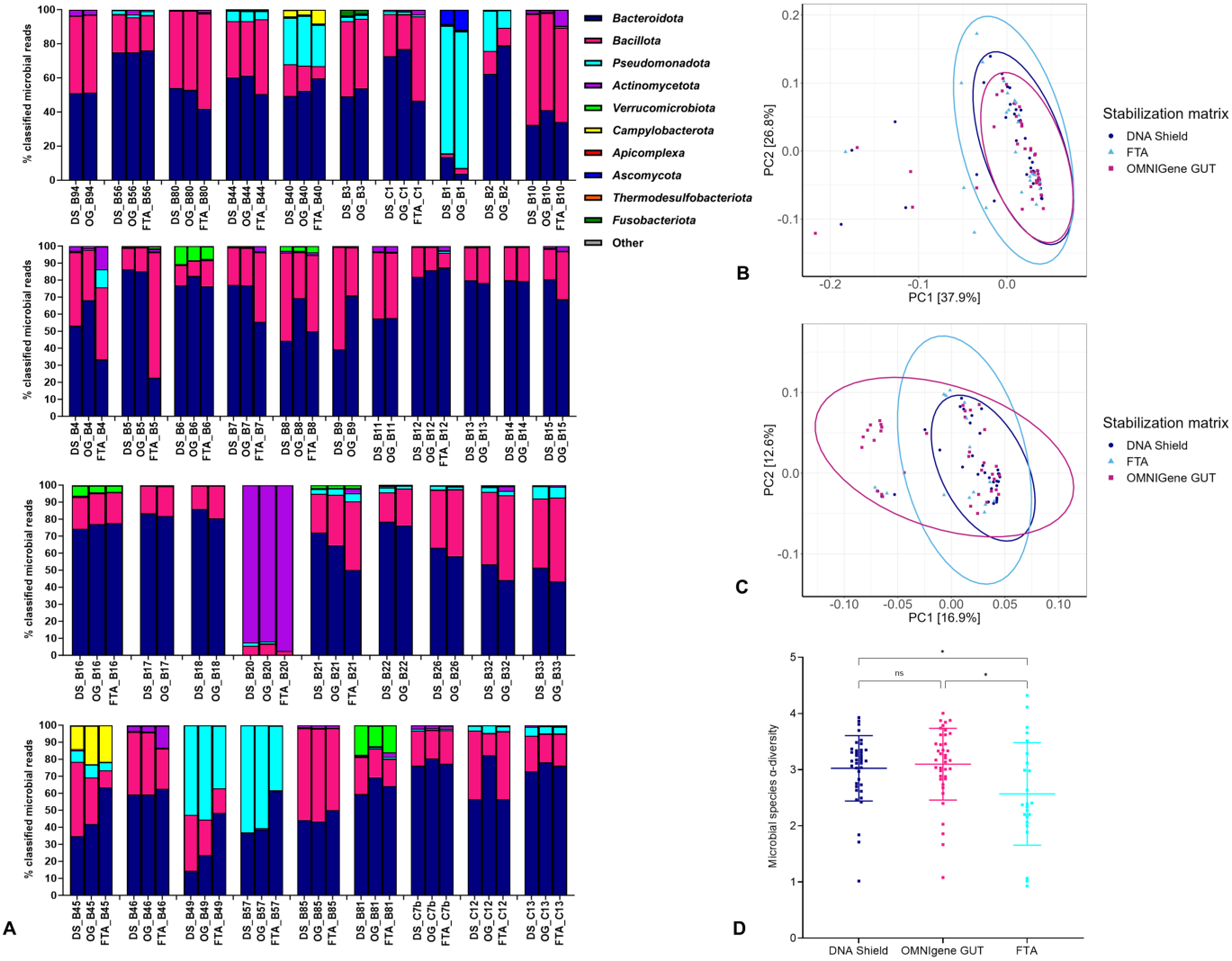

Figure 2 Microbial species composition. Proportion of classified microbial reads assigned to the ten most abundant microbial phyla (A). PCoA of the Bray-Curtis distance at the phylum level (B) and species level (C) with 95% confidence ellipses. The microbial species α-diversity (D). Samples that did not generate ≥100 classified microbial reads were excluded. DS=DNA Shield. OG=OMNIgene GUT. DS and OG n=39 FTA n=24. FTA, Flinders Technical Associate; PCoA, principal coordinate analysis.

will reduce false positives but may fail to detect low abundance pathogens, while a low threshold increases the risk of spurious identifications. Determining the limit of detection of *Cryptosporidium* from stool samples, such as through spiking negative stool samples with known oocyst concentrations[29] would assist in determining the suitable threshold moving forward.

A further challenge of CMg-based pathogen identification in the complex gut environment is distinguishing commensal flora from true pathogens, a limitation shared with PCR-based testing.[13 30] Development of CMg as an untargeted detection method would require a comprehensive list of enteric pathogens and associated virulence factors integrated into existing bioinformatic pipelines.

Storage methods also influenced the composition of the gut microbiome detected by CMg, with the FTA cards showing the poorest retention of species diversity, particularly species present at low abundances. These findings emphasise that careful consideration is required prior to stabilisation matrix selection according to the downstream diagnostic aims and further work is required to understand their impact on pathogen detection from stool samples by CMg.

CONCLUSIONS

This study reports the evaluation of CMg for the detection of the enteric parasite *C. hominis* from stool samples collected from UK military personnel stationed in Kenya during an outbreak of TD. The findings highlight the importance of stabilisation matrix selection for the preservation and detection of *Cryptosporidium* DNA from metagenomic data sets. This is particularly relevant where the pathogen DNA is present at low abundance and when prolonged ambient storage is unavoidable due to transport constraints or austere field conditions. The study also provides an improved understanding of CMg data interpretation for *Cryptosporidium* detection. The low read abundance observed suggests that detection of *Cryptosporidium* at any level may indicate infection, supporting the need to develop robust analytical thresholds for CMg-based diagnostics. More broadly, TD remains a significant cause of morbidity and operational disruption among deployed personnel. Although further technological development is required, CMg shows promise as a future deployable diagnostic capable of identifying causative pathogens and supporting improved outbreak response and targeted

treatment in austere environments to reduce the burden of TD in deployed populations.

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Contributors CH and RT are joint first authors. CH and ER participated in acquiring, analysing and interpreting the metagenomics and qPCR data. RT recruited personnel to the study and processed the faecal samples for PCR testing and for repatriation to the UK. TLV provided bioinformatics data processing and analysis. SS carried out the PCoA analysis of the composition of the microbial species. CH, RT and SAW wrote the manuscript, and all authors critically revised the manuscript and approved the final version. CH is the guarantor.

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Competing interests None declared.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by United Kingdom Ministry of Defence Research Ethics Committee (MODREC; 2076/MODREC/21). Participants gave informed consent to participate in the study before taking part.

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