Prevalence of gastrointestinal pathogenic bacteria in patients with diarrhoea attending Groote Schuur Hospital, Cape Town, South Africa

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Background. Diarrhoea due to gastrointestinal infections is a significant problem facing the South African (SA) healthcare system. Infections can be acquired both from the community and from the hospital environment itself, the latter acting as a reservoir for potential pathogenic bacteria.
Objectives. To examine the prevalence of a panel of potential diarrhoea-causing bacteria in patients attending a tertiary healthcare facility in Cape Town, SA.
Methods. Polymerase chain reaction (PCR) primers specific for *Clostridium difficile*, *Shigella* spp., *Salmonella* spp., *Klebsiella oxytoca*, enteropathogenic and enterohaemorrhagic *Escherichia coli* (EPEC/EHEC), *Staphylococcus aureus*, enterotoxigenic *Bacteroides fragilis* and *Campylobacter* spp. were used to screen total bacterial genomic DNA extracted from stool samples provided by 156 patients with diarrhoea attending Groote Schuur Hospital, Cape Town, SA.
Results. *C. difficile* was the most frequently detected pathogen (16% of cases) in the 21 - 87-year-old patient range, but was not present in samples from the 16 - 20-year-old range. *K. oxytoca* (6%), EPEC/EHEC strains (9%) and *S. aureus* (6%) were also detected. The remaining pathogens were present at low frequencies (0 - 2.9%), and the occurrence of mixed infections was 5%. The majority of non-*C. difficile*-related diarrhoeas were community acquired.
Conclusion. *C. difficile* was the main cause of infectious diarrhoea in the sampled patients, while *K. oxytoca* and EPEC/EHEC strains were present as relatively minor but potentially significant pathogens.

S Afr Med J 2015;105(2):121-125. DOI:10.7196/SAMJ.8654

Diarrhoea as a result of gastrointestinal tract infections is a significant problem facing much of Africa.[1] In 2000 alone, almost 4% of the deaths in South Africa (SA) were attributable to infectious diarrhoea, representing the fifth leading cause of years of life lost.[2] The causes of infectious diarrhoea are varied. Nosocomial infections are chiefly caused by *Clostridium difficile* and to a lesser extent by *Klebsiella oxytoca*, typically after antibiotic therapy, which allows the organisms to proliferate and cause disease.[3] Community-acquired diarrhoea resulting from person-to-person transmission or the consumption of contaminated and poorly prepared foodstuffs and water can be caused by a range of bacterial agents. These include several pathotypes of *Escherichia coli*, non-typhoidal *Salmonella* spp., *Shigella* spp., enterotoxigenic *Bacteroides fragilis*, *Campylobacter* spp. and *Staphylococcus aureus*, as well as several viruses (rotavirus, norovirus and adenovirus) and parasites (e.g. *Giardia lamblia* and *Entamoeba* spp.).[4]

Despite their potential to cause disease, there have been relatively few SA studies examining the prevalence of pathogenic micro-organisms in 'non-outbreak' situations, particularly in the hospital environment. In addition, the majority of surveillance studies have examined paediatric populations that are at increased risk of developing diarrhoeal disease. There is little information regarding the prevalence of potential pathogens in adults. The aim of this study was therefore to identify the prevalence of a selected panel of potential pathogenic bacteria in routine stool samples provided by patients with diarrhoea attending Groote Schuur Hospital (GSH), Cape Town, SA.

Methods

Sample collection and study participants

Stool samples (N=139) were collected as part of a larger study from in- and outpatients presenting to GSH with diarrhoea between March 2012 and March 2013. Patients <16 years old were excluded from the study. Samples were transferred to the National Health Laboratory Service (NHLS) unit at GSH, where they were stored at –20°C until further processing. The presence of blood in stool samples was assessed visually. Ethics approval was obtained from the University of Cape Town, Human Research Ethics Committee (HREC Number 310/2008).

Genomic DNA extraction

Total faecal genomic DNA was extracted from stool samples using the GXT Stool Extraction Kit (Hain Lifesciences, SA) and the GenoXtract automated extraction instrument following the manufacturer's instructions. The quantity and purity of the DNA were assessed using a Nanodrop 1000 instrument (Nanodrop, USA). The quality of the genomic DNA and the absence of polymerase chain reaction (PCR)

inhibitors were further assessed by PCR amplification of each sample using 50 ng template and the F27/R5 primer pair, which target the bacterial 16S rRNA gene.

Preparation of positive amplification controls

Bacterial-specific genomic DNA for use as a positive amplification control in the PCR screening amplifications was prepared from pure cultures of *C. difficile* (toxigenic isolate R20291), *C. jejuni* (a laboratory isolate provided by the NHLS, SA), *S. aureus* (a laboratory isolate) and *S. enterica* subsp. *enterica* serovar Typhimurium (ATCC 14028), using a genomic DNA extraction kit (ThermoScientific, USA) following the manufacturer's instructions. For the *B. fragilis bft* screening experiment, plasmids containing the cloned target region of the *bft-1* subtype (prepared in this study) and the *bft-2* subtype (kindly provided by Prof. Cynthia Sears), were used as positive amplification controls.[5] Plasmids containing cloned target regions of *K. oxytoca* (*pehX*), enteropathogenic and enterohaemorrhagic *Escherichia coli* (EPEC/EHEC) (*eaeA*) and the various pathogenic *Shigella* spp. (*invC*) were used as positive amplifications controls in the respective reactions.

Screening for potential pathogens

Total faecal genomic DNA (50 ng) prepared from each stool sample was used as template in a series of PCR amplifications using primers specific to each pathogen (Table 1). No template control reactions were included for each different primer set. PCR cycling parameters for the universal bacterial 16S rRNA gene, *C. jejuni*/*C. coli*, *S. aureus*, *Salmonella* spp., *Shigella* spp., *K. oxytoca* and EHEC/EPEC reactions were as follows: denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at primer-specific temperature for 30 seconds, and extension at 72°C for 1 minute, and finally 72°C for 7 minutes.

Table 1. Primers used in this study

Primer	Sequence (5′ - 3′)	Annealing temperature (°C)	Target/description	Size (bp)	Reference
F27	AGA GTT TGA TCI TGG CTC AG	55	Bacterial 16S rRNA gene	1 500	18
R5	ACG GIT ACC TTG TTA CGA CTT				
Tpi-F	AAA GAA GCT ACT AAG GGT ACA AA	65 - 55*	*C. difficile tpi* gene	230	3
Tpi-R	CAT AAT ATT GGG TCT ATT CCT AC				
TcdA-F	AGA TTC CTA TAT TTA CAT GAC AAT AT	65 - 55*	*C. difficile tcdA* gene	369/110†	3
TcdA-R	GTA TCA GGC ATA AAG TAA TAT ACT TT				
TcdB-F	GGA AAA GAG AAT GGT TTT ATT AA	65 - 55*	*C. difficile tcdB* gene	160	3
TcdB-R	ATC TTT AGT TAT AAC TTT GAC ATC TTT				
GBF-201	GAA CCT AAA ACG GTA TAT GT	62	Common forward primer		19
GBF-312	CCT CTT TGG CGT CGC		Reverse primer for *bft-1* gene	190	
GBF-322	CGC TCG GGC AACT AT		Reverse primer for *bft-2* gene	175	
GBF-334	TGT CCC AAG TTC CCC AG		Reverse primer for *bft-3* gene	287	
Peh-C	GAT ACG GAG TAT GCC TTT ACG GTG	59	*K. oxytoca pehX* polygalacturonase gene	344	20
Peh-D	TAG CCT TTA TCA AGC GGA TAC TGG				
eaeA-F	ATG CTT AGT GCT GGT TTA GG	58	EHEC/EPEC *E. coli eaeA* intimin gene	248	11
eaeA-R	GCC TTC ATC ATT TCG CTT TC				
CCCJ609-F	AAT CTA ATG GCT TAA CCA TTA	58	*C. jejuni*/*C. coli* 16S rRNA gene	854	21
CCCJ1442-R	GTA ACT AGT TTA GTA TTC CGG				
nuc-F	GCG ATT GAT GGT GAT ACG GTT	55	*S. aureus nuc* gene	280	22
nuc-R	AGC CAA GCC TTG ACG AAC TAA AGC				
invA-F	GTG AAA TTA TCG CCA CGT TCG GGC AA	64	*Salmonella spp. invA* gene	281	23
invA-R	TCA TCG CAC CGT CAA AGG AAC C				
SgenDF1	TGC CCA GTT TCT TCA TAC GC	60	*Shigella spp. invC* gene	875	24
SgenDR1	GAA AGT AGC TCC CGA AAT GC				

bp = base pairs.
*Touchdown PCR procedure: An initial annealing temperature of 65°C dropping to 55°C by 1°C per cycle for the first 11 cycles.
†The full-length *tcdA* gene fragment is 369 bp, whereas the truncated *tcdA* gene fragment in toxin A – B + *C. difficile* strains is 110 bp.

Multiplex PCR reactions were used to screen for *bft*-positive *B. fragilis* strains and toxigenic *C. difficile*. For the *B. fragilis bft* screening protocol, a common forward primer and three specific reverse primers that target the three different *bft* subtypes were used. The PCR cycling parameters were as follows: denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 62°C for 30 seconds and extension at 72°C for 1 minute, and finally 72°C for 7 minutes. For the *C. difficile* screening protocol, primers targeting the species-specific *tpi* gene as well as the two toxin genes *tcdA* and *tcdB* were used to identify toxigenic strains. A touchdown PCR procedure was employed. An initial denaturation at 95°C for 5 minutes was performed, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing for 30 seconds at temperatures decreasing from 65°C to 55°C (decreasing by 1°C per cycle for the first 11 cycles) and extension at 72°C for 30 seconds. A final extension step was then carried out at 72°C for 7 minutes. All reaction products were analysed by electrophoresis through 2% (w/v) agarose gels and imaged using a ChemiDoc EC imager (Bio-rad, SA). Positive controls (50 ng of pure genomic DNA from target strains or 50 ng of plasmid DNA containing the relevant target fragment) and negative controls containing no template were included in each PCR experiment.

Results

Basic demographic data

A total of 139 stool samples were analysed, of which 80 (57.6%) were from female patients. Patient ages ranged from 16 to 87 years, with the majority of the patients (73%) between 20 and 60 years of age.

Prevalence of selected pathogenic bacteria in diarrhoea samples

All samples showed the predicted 1.5 kb product when screened using the universal bacterial 16S rRNA gene primers (result not shown). Each of the primer sets gave the specific, expected product when used to amplify control target DNA under the study conditions (Fig. 1). The results of screening the study samples are summarised in Fig. 2. Of the 139 samples screened, 53 (approximately 38%) contained one or more of the target organisms possibly linked to the patient symptoms, while the remainder gave a negative result reflecting a diarrhoea of unknown origin. Toxigenic *C. difficile* was the most prevalent potential pathogen. It was found in approximately 16% of samples overall and in all age groups except the 16 - 20 years group. EPEC/EHEC *E. coli* were present in approximately 9% of cases and occurred across all age groups. *K. oxytoca* and *S. aureus* both occurred in 6% of samples, *Salmonella* spp., *Shigella* spp., and *bft*-positive *B. fragilis* together were present in approximately 8% of the samples, and *Campylobacter* spp. were not detected in any of them (Fig. 2). Mixed populations of potential pathogens were found in approximately 5% of the samples. Of the patient cohort, ten individuals (7%) showed evidence of blood in their stool. Two of these stools came from patients colonised by *Shigella* spp. and one from a patient colonised by *S. aureus*, while the remaining seven bloody stools did not contain any of the pathogens included in the screening procedures.

Discussion

Routine surveillance of bacteria that are known agents of infectious diarrhoea is very seldom carried out for organisms other than *Shigella* spp. and non-typhoidal *Salmonella* spp. In this study, we sought to examine the prevalence of other potential bacterial pathogens present in diarrhoea samples from patients attending a hospital in Cape Town. The results suggested that *C. difficile* was the most prevalent pathogen among patients in the 21 - 87-year-old range, with the rest of the selected potential pathogens making up a relatively small percentage of the cases.

C. difficile is a frequent cause of nosocomial diarrhoea, accounting for up to 20% of such cases worldwide.[3] There have also been reports suggesting that the prevalence of community-acquired cases of *C. difficile* is increasing.[6] Importantly, there are few data on the prevalence of *C. difficile* in SA. A previous study, also carried out at GSH but employing an enzyme-immunoassay (EIA)-based technique, determined a prevalence of 9.2% for toxigenic *C. difficile* in patients with diarrhoea.[7] It is possible, however, that some *C. difficile* cases were overlooked in this earlier study, as EIA-based tests have been reported to suffer from inferior diagnostic sensitivity.[8]

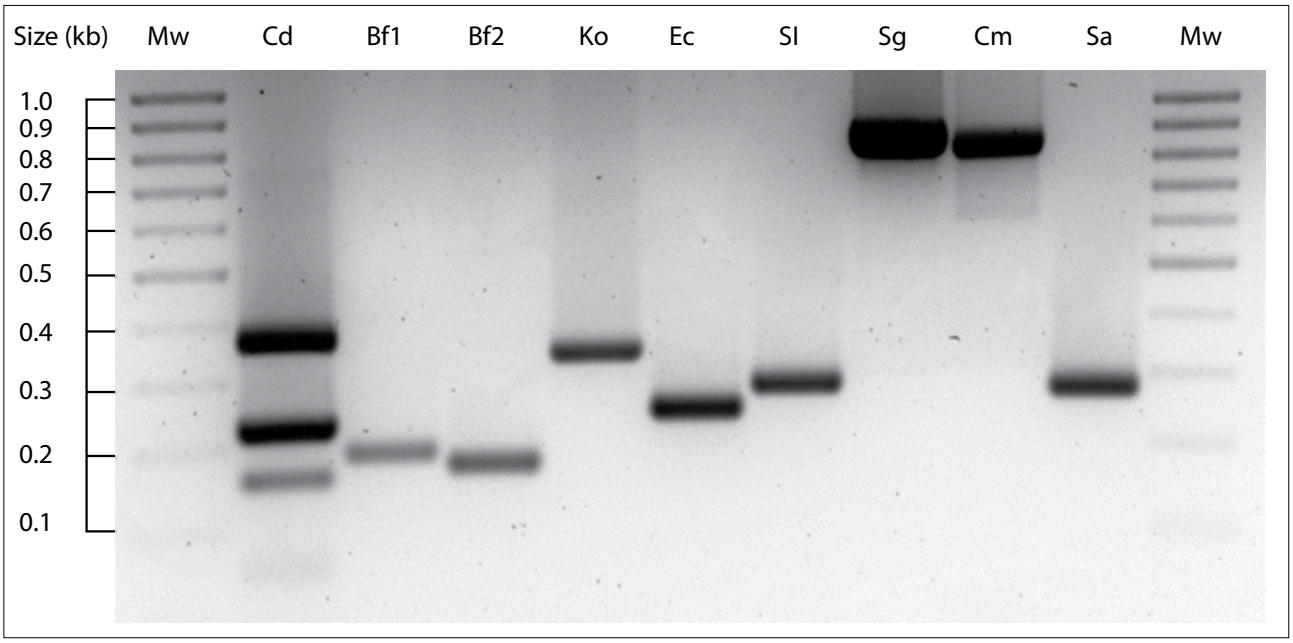

Fig. 1. Positive control reactions for each primer set. Lanes: Mw (100 bp DNA ladder), Cd (C. difficile), *Bf1* (B. fragilis bft-1), *Bf2* (B. fragilis bft-2), *Ko* (K. oxytoca), *Ec* (*EHEC/EPEC* E. coli), *Sl* (Salmonella *spp.*), *Sg* (Shigella *spp.*), *Cm* (C. jejuni/C. coli), *Sa* (S. aureus).

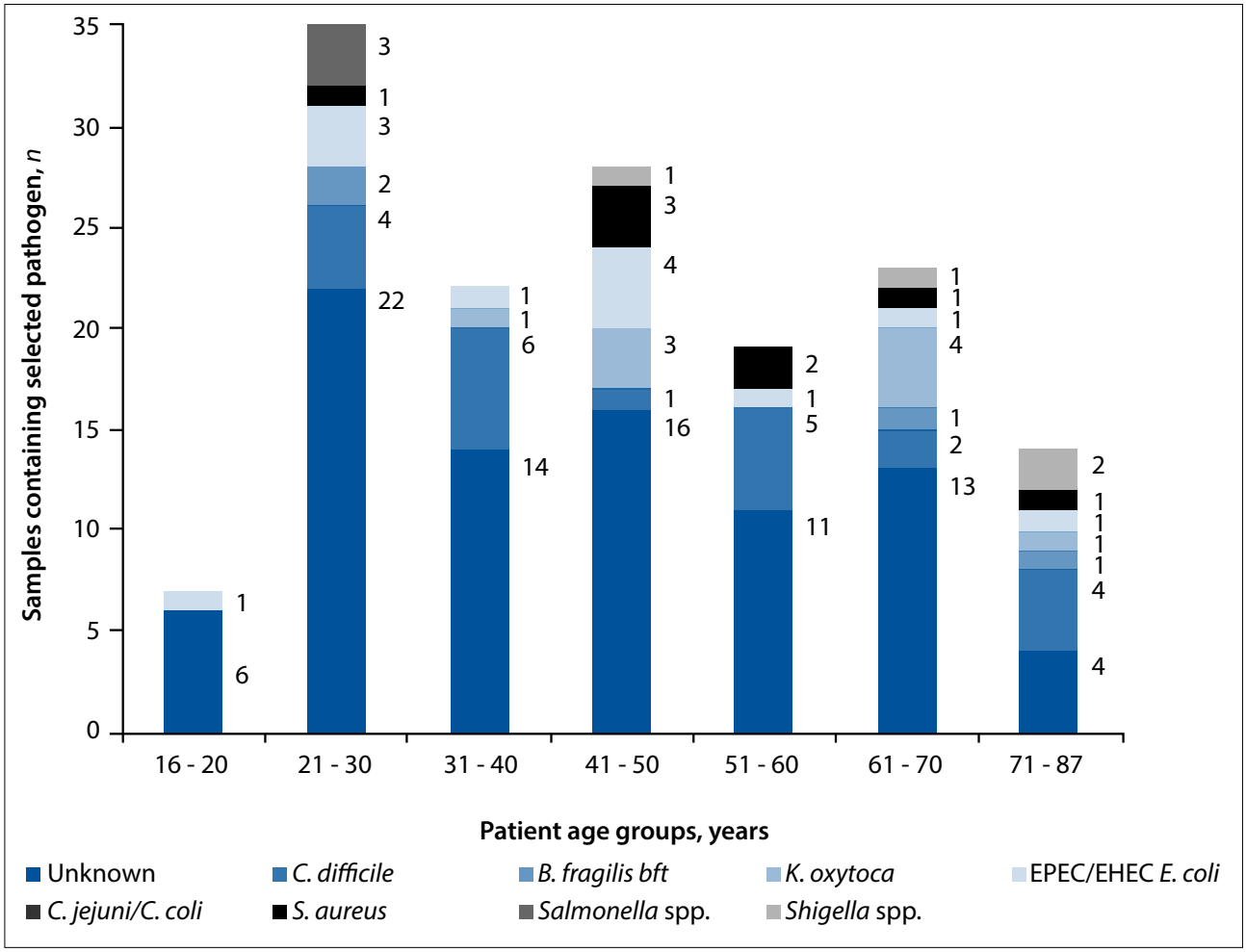

Fig. 2. Prevalence of selected pathogenic bacteria in diarrhoea samples. The numbers of samples containing the selected pathogen are given next to the histograms for each age group. Samples that did not contain any of the selected pathogens investigated in the study were reported as 'Unknown'.

The only other PCR-based analysis reported a prevalence of 11.4% of toxigenic *C. difficile* in individuals with diarrhoea in the Vhembe district, suggesting that *C. difficile*-related infections are an important but possibly under-reported cause of diarrhoea in SA.[9] A more detailed analysis of the prevalence and epidemiology of *C. difficile* at GSH as well as a comparison of various diagnostic testing modalities is currently being prepared for publication.

Pathogenic *E. coli* are traditionally divided into several different pathotypes. While enteroaggregative *E. coli* are increasingly recognised as an important cause of diarrhoea in both Africa and the rest of the world, their broad genetic diversity means that in order to detect them using a PCR-based screening method, several primer combinations detecting different targets need to be employed.[10] We therefore limited our screening procedure to detect EPEC and EHEC strains, both of which have been known to cause outbreaks of diarrhoea in Africa and can be detected by the presence of the *eaeA* gene.[11] In the current study, EPEC and EHEC strains were present in just under 9% of the diarrhoea cases. Of these cases, 8/12 (67%) developed prior to hospital admission, suggesting that the majority of the *E. coli* infections were community acquired. Blood in the stool was not evident in any of the patients colonised by EPEC/EHEC strains. A previous study by Bisi-Johnson *et al.*[12] identified EPEC and EHEC strains in approximately 13% of patients with diarrhoea attending a tertiary hospital in the Eastern Cape Province, SA. However, that study had a large proportion of young patients (30% of the patients were between the ages of 7 and 13), who are at increased risk of developing *E. coli*-related diarrhoea.

K. oxytoca has been implicated as a cause of antibiotic-associated haemorrhagic colitis. Clinical isolates have been shown to constitutively produce β-lactamases, which confer resistance to both amino- and carboxypenicillins and allow the organism to survive antibiotic therapy and initiate infection.[13] A small proportion of healthy individuals (1.6%) are asymptomatic carriers of *K. oxytoca*, although the carriage rate in non-symptomatic patients attending GSH is currently unknown.[14] None of the samples positive for *K. oxytoca* showed evidence of blood in the stool, suggesting that *K. oxytoca* was not a major cause of diarrhoea among the patients examined.

Non-typhoidal *Salmonella* spp. and *Shigella* spp. are responsible for a significant number of cases of diarrhoea in Africa and are regarded by the World Health Organization as organisms of global significance.[1,15] *Shigella* spp. in particular have been known to cause outbreaks of bloody diarrhoea among adults in much of the developing world.[4] Of the four patients colonised by pathogenic *Shigella* spp., two showed strong evidence of blood in the stool. Although occasional nosocomial outbreaks have been reported in SA, both pathogens are predominantly acquired through the ingestion of contaminated food and water or from person-to-person transmission via the faecal-oral route. In the current study, all the diarrhoea cases in which either pathogen was present

were community acquired. However, the combined prevalence of approximately 5% suggests that neither pathogen was a major cause of diarrhoea in the sample group.

B. fragilis is a human gut commensal that is also able to cause opportunistic invasive infections. In addition, certain strains also produce a metalloprotease enterotoxin encoded by the *bft* gene that enables the bacterium to cause diarrhoea.[5] Screening of the samples in the current study using primers that target all three subtypes of the *bft* gene revealed a low prevalence of *bft*-positive *B. fragilis* strains.

Enterotoxin-producing *S. aureus* is a fairly rare cause of diarrhoea, but is of particular significance in the hospital environment owing to its role in postoperative infections. There have also been reports that many samples from patients with antibiotic-associated diarrhoea that were positive for enterotoxin-producing *S. aureus* were also positive for *C. difficile*.[16] In the current study, 5/8 (62.5%) of the samples positive for *S. aureus* also harboured at least one of the other potential pathogens that were included in the screen. *C. difficile* was co-present in three of these samples and is presumed to be the main cause of diarrhoea in these patients. It is not clear from these results whether the presence of *S. aureus* promoted colonisation by other potential pathogens or vice versa.

In developing countries, *Campylobacter* spp., predominantly *C. jejuni* and *C. coli*, are the most common bacterial cause of diarrhoea in babies in the first year of life.[4] A previous study by Samie *et al.*[17] reported prevalences of 12.5% and 7% for *C. jejuni* and *C. coli*, respectively, among patients between the ages of 0 and 88 attending hospitals in the Venda region of SA. However, in the patient group examined in our study, it was not possible to detect either species in the stool samples using the described primer set, the specificity of which was validated using the *C. jejuni*-positive control DNA.

An initial screening of the purified DNA using universal primers targeted to the bacterial 16S rRNA gene yielded positive products for each of the samples, indicating that the quality of the extracted DNA was suitable for PCR analysis. However, it is possible that in cases where targets were not abundant in the samples, these may not have been detected by the individual PCR screening experiments. In addition, other bacterial strains, parasites and viruses as well as non-infectious factors (e.g. direct gut toxicity of administered antibiotics) were not included in this pilot study and are reflected as being of unknown origin (Fig. 2). These might be contributing to the diarrhoea cases observed and should be included in further studies. In particular, protocols to detect the prevalence of rotavirus (which has an RNA not a DNA genome) should be used during stool analysis. Nevertheless, the results presented here suggest that, in addition to *C. difficile*, other bacterial pathogens such as EPEC/EHEC strains that are not routinely screened for in the hospital setting may be responsible for a number of episodes of diarrhoea among adults in SA, and this warrants further investigation.

Acknowledgements. The authors acknowledge funding from the National Research Foundation of South Africa. BK acknowledges bursary funding from the Carnegie Corporation and the Claude Leon Foundation. The authors are grateful to Hain Lifescience for the loan of the GenoXtract automated extraction instrument and extraction kits for use in the study, and to the staff of the NHLS at GSH for assistance in the collection of samples.

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Accepted 9 December 2014.