JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Detection of *Campylobacter* species in stool specimens from patients with symptoms of acute flaccid paralysis in South Africa

Mandile Samantha Thobela[1,2], Anthony Marius Smith[1,2], Shelina Moonsamy[3], Heleen du Plessis[3], Nevashan Govender[4], Karen Helena Keddy[1,2]

[1] Division of the National Health Laboratory Service, Centre for Enteric Diseases, National Institute for Communicable Diseases, Johannesburg, South Africa
[2] Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa
[3] Division of the National Health Laboratory Service, Centre for Vaccines and Immunology, National Institute for Communicable Diseases, Johannesburg, South Africa
[4] Division of Public Health Surveillance and Response, National Institute for Communicable Diseases, a division of the National Health Laboratory Service, Johannesburg, South Africa

Abstract

Introduction: Guillain-Barré Syndrome (GBS) is an autoimmune disease characterized by acute or subacute symmetrical ascending motor weakness, areflexia, and mild-to-moderate sensory abnormalities. *Campylobacter jejuni* is reported to be the most common bacterium associated with GBS cases. Despite the eradication of polio, the number of reported GBS cases remains considerably high in South Africa with the causative agents not being well described. Methodology: The aim of the study was to investigate the proportion of *Campylobacter* spp. detected in stool specimens from patients with symptoms of acute flaccid paralysis (AFP). Stool specimens from patients presenting with AFP, that were negative for polio and non-polio enteroviruses (NPENT), were processed and screened for the presence of *Campylobacter* spp. using quantitative PCR (qPCR). Results: Of the 512 stool specimens screened between October 2014 to December 2015, 12% (62/512) were positive for *Campylobacter* spp. Of these 62 *Campylobacter* infections: 77.4% (48/62) was *C. jejuni*; 19.4% (12/62) was *Campylobacter coli*; 3.2% (2/62) was mixed infections of *C. jejuni* and *C. coli*. Conclusions: True association of the disease with *Campylobacter* spp. will enable the proportion of *Campylobacter*-induced GBS to be better described in South Africa; this can only be done through systematic studies that include bacterial culture and serology together with molecular methodologies.

Key words: *Campylobacter*; acute flaccid paralysis, AFP; Guillain-Barré syndrome; GBS; quantitative PCR; qPCR.

J Infect Dev Ctries 2018; 12(7):542-549. doi:10.3855/jidc.9795

(Received 29 September 2017 – Accepted 31 May 2018)

Introduction

Guillain-Barré Syndrome (GBS) is an autoimmune disease associated with a history of a preceding bacterial or viral infection [1-3]. With polio almost eradicated globally, GBS has now become the most common cause of acute flaccid paralysis (AFP) with an annual incidence of 0.6–4.0 cases per 100000 population [4,5]. *Campylobacter* spp., particularly *Campylobacter jejuni*, have been described as one of the leading causes of human gastroenteritis [1-5]. Pathogenic features of *C. jejuni* influencing the development of GBS have been described, with *C. jejuni* infections reportedly constituting the majority of GBS cases [1-5]. GBS, which develops after *C. jejuni* infection, has been investigated and reported in a number of studies, rendering characteristics of *C. jejuni* strains subject to renewed interest in association with the pathogenesis of GBS [4,6-9].

C. jejuni-induced GBS is the most common and severe sequela of *Campylobacter* gastroenteritis, that is characterized by acute or subacute symmetrical ascending motor weakness, areflexia, and mild-to-moderate sensory abnormalities [4,8,10-12]. The disease is attributed to sialylated surface polysaccharide structures and flagella of some *C. jejuni* strains, which are thought to be responsible for the ganglioside mimicry and antibody formation leading to GBS [13-16]. Although gastroenteritis caused by *C. jejuni* is common, the risk of developing GBS after *C. jejuni* infection is rare, with reported incidences of less than 1% (1:1000 cases develop GBS) [4,10,12]. *C. jejuni* isolation from stool cultures of GBS patients ranges from 8% to 50% [4,13]. The limitation in associating

the development of GBS following *C. jejuni* enteritis is that the bacteria are usually eliminated from the body by the time a patient present with GBS. The elimination of the bacteria from the body occurs within 16 days of infection and before the onset of acute neuromuscular paralysis, which usually occurs 10 days to 3 weeks after the onset of diarrhoea [1,10,14-17]. *Campylobacter*-induced GBS cases also go unrecognized because *Campylobacter* is not routinely diagnosed in resource poor settings, and when diagnosis is made, the use of antimicrobials also compromises isolating *Campylobacter* spp. in culture [14].

The Penner heat-stable (HS) serotypes of *C. jejuni* strains that are attributed to GBS include serotype HS:19 which has been reported to occur globally; and serotype HS:41 which reportedly is restricted to Cape Town, South Africa [8,11,12,18-20]. During a five year study period (January 2005 to December 2009) a total of 1 501 AFP cases were reported in South Africa, of which 67.2% (1009/1501) were children aged < 5 years of age and 54.3% (815/1501) were male [21]. The overall occurrence of non-polio AFP was 1.8 AFP cases per 100000 population of individuals aged < 15 years in South Africa [21]. The detection rate of AFP in South Africa showed an increase from when the study commenced; from 1.6 cases in the year 2005 to 2.1 cases per 100000 (< 15 years) in the year 2009 [21]. For those cases where a diagnosis could be made GBS was the most common cause of AFP in South Africa, accounting for 42.7% (321/751). Despite the implementation of the AFP surveillance system in South Africa in 1997 to monitor progress towards eradication of poliomyelitis, there is insufficient published studies describing the epidemiology of AFP in South Africa.

It is plausible that findings from the few published studies concerning AFP signify that non-polio AFP is an increasing problem that is associated with increased morbidity in South Africa and as a result, the aetiological agents resulting in GBS should be determined. To date, there remain research gaps regarding the prevalence of *Campylobacter* in stool specimens of AFP patients and the proportion of *Campylobacter*-induced GBS in South Africa is unknown. We have initiated preliminary investigations to address these gaps. In our present study, for the period of October 2014 to December 2015, stool specimens from AFP case patients in South Africa, confirmed as negative for polio virus, were screened and investigated for the presence of *Campylobacter*.

Methodology

Ethical standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees of human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals. Ethical clearance to do surveillance was obtained from the Wits Human Research Ethics Committee, in the name of Mandile Samantha Thobela, on the 25th of September 2014 (Clearance Certificate No: M140750).

Case definitions

The case definition of a suspected polio case is: (1) any case of AFP, including GBS, in a person under 15 years of age for any reason other than severe trauma, (2) any paralytic illness in a person of any age in which polio is suspected.

Stool specimens

Stool samples were collected following the World Health Organization (WHO) guidelines for AFP surveillance [22-24] and submitted to the Regional Polio Reference Laboratory, Centre for Vaccines and Immunology, National Institute for Communicable Diseases (NICD), for processing and testing. For optimal isolation, two stool samples, 24 to 48 hours apart and within 14 days of onset of paralysis were collected from each case and transported to the testing laboratory at a temperature of 2°C to 8°C. Between October 2014 to December 2015 a total of 1337 stool specimens (n = 226 October to December 2014; n = 1111 January to December 2015) were submitted to the Regional Polio Reference Laboratory. Of the 1111 stool specimens that were submitted in the year 2015, four (0.4%; n = 4/1111) tested positive for polio virus (Sabin vaccine strain) and these were omitted from the study. Specimens that were positive for non-polio enteroviruses (NPENT) were also omitted from the study; these were 13% (n = 29/226) in the year 2014 and 13% (n = 142/1111) in the year 2015.

DNA extraction from stool specimens

Frozen polio-negative stool specimens were processed to extract bacterial DNA using the QIAamp Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions. DNA

extractions were used as template DNA in quantitative PCRs (qPCR).

To validate the extraction efficiency of the QIAamp Fast DNA Stool Mini Kit, a stool specimen from a healthy individual was spiked with 0.5 McFarland bacterial lysate in normal saline (Diagnostic Media Products, Johannesburg, Republic of South Africa) prepared from *C. jejuni* (strain C12.2 - WHO EQA 2012) and *C. coli* (strain C9.1 - WHO EQA 2009) control strains respectively (100 µL bacterial lysate into ~200 mg stool specimen), following which DNA was extracted as outlined by the extraction protocol. Extracted DNA from spiked stool specimens yielded the expected PCR results for each control strain.

Multiplex qPCR

A previously described multiplex qPCR targeting genes that are specific for *C. jejuni* and *C. coli,* as well as the *16SrRNA* gene unique to all *Campylobacter* spp. (Table 1) was used in our current study [25-27]. Multiplex qPCRs were performed in 50 µL reactions and included the following components: 25 µL TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, USA); 12 µL internal positive control (IPC) mix (Applied Biosystems, Foster City, USA; 7.5 µM *mapA* primer with 1.5 µM *mapA* probe; 15 µM *ceuE* primer with 3 µM *ceuE* probe; and 15 µM *16SrRNA* primer with 3 µM *16SrRNA* probe (Inqaba Technologies, Pretoria, South Africa, (primers); and Roche, Johannesburg, South Africa, probes); 7 µL template DNA; and 3 µL of deionized water. The PCR reaction assay was carried out on the Applied Biosystems 7500 real-time PCR machine (Applied Biosystems, Foster City, USA) with cycling conditions set as follows: 50˚C for 2 minutes (1 cycle); 95˚C for 10 minutes (1 cycle), followed by 95˚C for 15 seconds and 60˚C for 1 minutes (40 cycles). The results were viewed and analyzed using the AB 7500 System SDS Software. PCR positive results were determined when a PCR result showed a typical PCR amplification curve (sigmoidal curve) together with C_t values of 15-38.

Statistical analysis of data

Statistical analysis was performed using Epi Info 7 (Centers for Disease Control & Prevention, Atlanta, USA). Testing for statistical significance was performed using Chi-squared and Fisher's Exact tests from which a p-value of < 0.05 was taken to be statistically significant.

Results

A total of 512 (44%; $n = 512/1162$) polio-negative stool specimens (first stool sample) were found and collected for *Campylobacter* spp. screening between October 2014 and December 2015 ($n = 16$ months) (Table 2). The age range of case patients in the study was < 1-67 years, with a median age of 4 years [Interquartile range (IQR) = 2-12]. Fifty-three per cent (53%; 271/512) of the specimens were from males, 47% (239/512) from females, and sex for < 1% (2/512) of the specimens was not specified.

Detected species of Campylobacter

Campylobacter spp. were detected in 12% (62/512) of the screened stool specimens using qPCR. *C. jejuni* was the most common species detected (77.4%; 48/62), followed by *C. coli* (19.4%; 12/62), and mixed infections of *C. jejuni* and *C. coli* (3.2%; 2/62) (Table 2).

Sex distribution of AFP patients with Campylobacter positive stool specimens

Campylobacter spp. were more frequently detected in female patients, with females and males accounting

Table 1. *Campylobacter* target gene sequences and primer/probe sequences [25-27].

Species	Target gene	PCR primer / probe	Primer/probe sequence (5'-3')	Product size (base pair)
C. jejuni	*mapA*	*mapA*-F1	CTGGTGGTTTTGAAGCAAAGATT	95
		mapA-R1	CAATACCAGTGTCTAAAGTGCGTTTAT	
		mapA-probe1	**6FAM** - TTGAATTCCAACATCGCTAATGTATAAAAGCCCTTT - **BHQ1**	
C. coli	*ceuE*	*ceuE*-F1	AAGCTCTTATTGTTCTAACCAATTCTAACA	103
		ceuE-R1	TCATCCACAGCATTGATTCCTAA	
		ceuE-probe1	**Cy5** - TTGGACCTCAATCTCGCTTTGGAATCATT - **BBQ**	
Non-*C. Jejuni / C. coli*	*16SrRNA*	Camp -F2	CACGTGCTACAATGGCATAT	108
		Camp-R2	GGCTTCATGCTCTCGAGTT	
		Camp-probe P2	**Cy3** - CAGAGAACAATCCGAACTGGGACA - **BBQ**	

for 55% (34/62) and 45% (28/62) respectively; however, this finding was not significant (p-value = 0.22). At species level, females accounted for 54% (26/48) of the detected *C. jejuni* and males accounted for 46% (22/48) (p-value = 0.22). *C. coli* was detected equally from both females and males, accounting for 50% (6/12) in both females and males. Mixed infections of *C. jejuni* and *C. coli* were detected in females only.

Age distribution of AFP patients with Campylobacter positive stool specimens

The detection of *Campylobacter* spp. was notable in the < 1-2 years' age group (37%; 23/62), 3-5 years age group (32%; 20/62), and the 6-9 years age group (18%; 11/62) (Table 2). The majority of the detected species of *Campylobacter* in these age groups were *C. jejuni*, accounting for ≥19%. Other age groups from which *Campylobacter* was detected (at levels of ≤ 6%) were those aged 10-19 years (6%; 4/62), 40-49 years (2%; 1/62), and those with unknown age groups (5%; 3/62).

Table 2. Stool specimens from polio-negative AFP case patients screened for the presence of *Campylobacter* species during October 2014 to December 2015.

Characteristic	Total submitted (%)	Total positive (%)	*Campylobacter* spp. (%)		
			C. jejuni	*C. coli*	[ᵑ]Mixed
Specimen type					
Stool	512	62 (12)	48 (77.4)	12 (19.4)	2 (3.2)
Age (years)					
Median ([*]IQR)	4(2-12)				
Range	< 1-67				
Age category (years)					
0-2	140 (27)	23 (37)	20 (42)	3 (25)	0
3-5	188 (37)	20 (32)	13 (27)	5 (42)	2 (100)
6-9	85 (17)	11 (18)	9 (19)	2 (17)	0
10-19	68 (13)	4 (6)	3 (6)	1 (8)	0
20-29	6 (1)	0	0	0	0
30-39	4 (< 1)	0	0	0	0
40-49	3 (< 1)	1 (2)	1 (2)	0	0
50-59	2 (< 1)	0	0	0	0
≥ 60	1 (< 1)	0	0	0	0
[¥]Unk	15 (3)	3 (5)	2 (4)	1 (8)	0
Gender					
Female	239 (47)	34 (55)	26 (54)	6 (50)	2 (100)
Male	271 (53)	28 (45)	22 (46)	6 (50)	0
Unk	2 (< 1)	0	0	0	0
[£]p-value		0.22			
Province					
Eastern Cape	94 (18)	20 (32)	17 (35)	3 (25)	0
Free State	21 (4)	3(5)	3 (6)	0	0
Gauteng	104 (20)	15 (24)	11 (23)	3 (25)	1 (50)
KwaZulu-Natal	93 (18)	8 (13)	7 (15)	1 (8)	0
Limpopo	64 (13)	9 (15)	5 (10)	4 (33)	0
Mpumalanga	63 (12)	1 (2)	1 (2)	0	0
North West	24 (5)	1 (2)	0	0	1 (50)
Northern Cape	11 (2)	0	0	0	0
Western Cape	36 (7)	4 (6)	4 (8)	0	0
Unk	2 (< 1)	1 (2)	0	1 (8)	0
[ᵠ]**Annual quarter**					
Jan-Mar	107 (21)	18 (29)	13 (27)	3 (25)	2 (100)
Apr-Jun	106 (21)	5 (8)	3 (6)	2 (17)	0
Jul-Sep	122 (24)	15 (24)	11 (23)	4 (33)	0
Oct-Dec	164 (32)	21 (34)	19 (40)	2 (17)	0
Unk	13 (3)	3 (5)	2 (4)	1 (8)	0

[*]Interquartile range (IQR).; [£]p-value < 0.05 statistically significant; [ᵠ]Annual quarter: January (Jan), March (Mar), April (Apr), June (Jun), July (Jul), September (Sept), October (Oct); and December (Dec); [¥]Unknown (Unk); [ᵑ]Mixed (mixed infections of *C. jejuni* and *C. coli*).

Annual distribution of positive cases of Campylobacter

Collectively (2014-2015), the majority of *Campylobacter* positive specimens were detected during the fourth quarter (October to December) of the year (34%; 21/62), followed by the first quarter (January to March) of the year (29%; 18/62), and lastly the third quarter (July to September) of the year (24%; 15/62). *Campylobacter* spp. were least detected during the second quarter (April to June) of the years (8%; 5/62).

Provincial distribution of detected species of Campylobacter

The Eastern Cape Province (32 %; 20/62), Gauteng Province (24%; 15/62), Limpopo Province (15%; 9/62), and KwaZulu-Natal Province (13%; 8/62) had the highest number of detected *Campylobacter* spp. (Table 2). In the Eastern Cape Province, 85% (17/20) of the detected *Campylobacter* spp. were *C. jejuni*, as well as 73% (11/15) of the detected *Campylobacter* spp. in the Gauteng Province. Overall, *C. jejuni* was the most common species of *Campylobacter* detected in all provinces. In the Northern Cape Province, no *Campylobacter* was detected.

Discussion

In our study, *Campylobacter* was detected in 12% (62/512) of the screened stool specimens using qPCR. This qPCR method was selected over culture methods because the DNA samples used in the study were extracted from 'old' frozen stool specimens. We only received frozen stool samples 1-2 weeks post-date of specimen collection, so an attempted culture of *Campylobacter* would very likely have failed. *Campylobacter* is a fastidious organism that is difficult to culture even from fresh stool samples [32-33]. It is well documented that in studies where qPCR was used as a diagnostic tool, more *Campylobacter* species were detected than with culture methods [33].The most common detected species of *Campylobacter* was *C. jejuni* (77.4%), an organism associated with GBS [4,10,14,15]. *C. coli* was less frequently detected (19.4%), and mixed infections of *C. jejuni* and *C. coli* were rare (3.2%). While *C. jejuni* is a well-documented elicitor of GBS, it is unknown whether *C. coli* elicits GBS [14]. These findings suggest that other species of *Campylobacter*, although not yet recognised, may be important in the pathogenesis of GBS [14,28]. In developing countries, asymptomatic infections with *Campylobacter* occur due to recurrent *Campylobacter* infections as residents are repeatedly exposed to these pathogens [9,10]. This may partly explain the detection of species of *Campylobacter* from AFP patients which have not yet been documented as elicitors of GBS, such as the detection of *C. coli* in our study. Other pathogens documented to cause GBS [6] may also be the underlying cause of the higher number of AFP patients in our study from whom *C. jejuni* was not detected (88%; 452/512).

Patient sex distribution has in almost all reports shown males to be slightly more frequently associated with *Campylobacter*-induced GBS in comparison to females [4,16,29,30]. Most of these studies investigating *Campylobacter*-induced GBS were conducted in developed countries. A study conducted in Egypt showed the frequent association of GBS with males which coincides with what is observed in developed countries [9]. However, in our study, although not statistically significant (p-value = 0.22), *C. jejuni* was more commonly detected in females (54%) compared to males (46%). According to Frazer *et al*. [31], females and males are equally susceptible to polio infections. Although polio-negative specimens that were screened for the presence of *Campylobacter* in our study were more frequently from males (53%; 271/512) than females (47%; 239/512), the rate of *Campylobacter* detection was more in females than males. However, because our study period was short and unsystematic, we could not report with certainty that sex distribution of AFP patients with *Campylobacter* positive specimens was not comparable with previous reports of *Campylobacter*-induced GBS being more common in males than females [4,16,19].

Patients from whom *Campylobacter* was frequently detected were those aged < 1-2 years (37%), 3-5 years (32%), and those aged 6-9 years (18%). *Campylobacter* spp. were not detected in all age groups represented in this study (Table 2). Other age groups with relatively few specimens from whom *Campylobacter* was detected were those aged 10-19 years (6%), and the single adult case (40-49 years age group) accounting for 2%, as well as the 5% represented by patients of unspecified age groups. According to Khuzwayo *et al*. [21], a case definition of AFP in South Africa is defined as any child of less than 15 years of age presenting with acute onset of focal weakness or paralysis which is characterized as flaccid, including GBS, without any other obvious cause. *Campylobacter* positive cases in our current study thus reflected a predominance of children and teenagers, who are individuals identified by the case definition for AFP surveillance. The study conducted in Egypt by Wierzba *et al*. [9], showed the incidence of *C. jejuni*-induced GBS to be higher in children from developing countries; an observation

which contrasts reports from developed countries where the prevalence of GBS is commonest among adults [16,29,30]. According to Nyati and Nyati [4], patients of any age may develop GBS. Reports have shown, however, the incidence of GBS to increase with age, and according to Islam *et al*. [30], *C. jejuni*-induced GBS incidence is lower in children. Nevertheless, results from our study coincided with that of Wierzba *et al*. [9], mainly because AFP cases are generally observed amongst young children globally and this observation influenced the case definition of AFP patients (< 15 years) included in surveillance. Given that gastroenteritis due to *C. jejuni* infections are hyperendemic among young children (< 5 years) in tropical resource-poor settings, it is expected that the prevalence of *C. jejuni*-induced GBS would be prevalent among individuals of < 5 years of age. Other researchers, using a broader case definition that includes all age groups for GBS surveillance, have published reports of bimodal distribution of GBS cases by age, with more cases identified in young adults (15-30) and the elderly (significantly higher) [4,16,29,30].

Most GBS cases are reported to be sporadic; however, a seasonal trend has been reported by some studies which describe a surge in the summer months according to Nachamkin *et al*. [16], and Dimachkie and Barohn [3]. Studies describing the precise seasonal distribution of GBS are still lacking, however, summer peaks of GBS have been reported in China, Mexico, and Spain [4,16]. In 2008, Wierzba and colleagues reported GBS lacked a marked seasonal variation in Egypt [9]. Although the majority of the specimens that tested positive for *Campylobacter* were submitted during fourth quarter (34%; 21/62) and the first quarter of the year (18%; 29/62) which are characterized by warmer months; seasonal trends of GBS in South Africa have not yet been documented. Our current study period was too short for us to define seasonal trends for positive cases.

The majority of *C. jejuni* detected from stool specimens were submitted from the Eastern Cape Province and Gauteng Province (Table 2). The reduced detection rate of *C. jejuni* from the respective provinces may be largely compromised by the fact that many *Campylobacter*-induced GBS cases go unrecognized because by the time patients present with AFP, under this scenario, the bacteria may have already been eliminated from the body and so would go undetected [14,17,21]. According to Nechamkin [14], history of antimicrobial use from patients with GBS should be clarified, as antimicrobial use in patients will have a marked challenge when isolating the underlying pathogen causing GBS.

Study limitations

The epidemiological element of the study was incomplete; patient history of preceding diarrhoea and antibiotic treatment was unknown. The *Campylobacter* detected may not have been associated with diarrhoea, but could have been associated with asymptomatic carriage. The AFP analysis was also limited by the absence of a non-AFP control group. This made interpretation of these findings challenging, because the background prevalence of *Campylobacter* in the population was unknown. Furthermore, we could not definitively link GBS to the detection of *C. jejuni*, as we did not look for those serotypes of *C. jejuni* specifically associated with GBS. We elected to use PCR to identify *Campylobacter* from stool to improve our sensitivity of diagnosis and because yields of *Campylobacter* from stool culture are frequently low [32]. Culture, accompanied by Penner serotyping would have better characterized the isolates, permitting better associations between the detection of *C. jejuni* and AFP patients in our study. Stool collection methods for the AFP surveillance did not permit culture as an option. The majority of the patients enrolled in the AFP Surveillance Program were aged < 15 years, according to the national and WHO guidelines: this limited the opportunity to identify *Campylobacter* as a cause of GBS in older age groups. More isolates were represented by the fourth quarter of the year (October to December) because collection of stool specimens began during October of the year 2014, thus affecting interpretation of seasonality.

Conclusions

The unsystematic nature of this study design cannot definitively confirm the degree of association of GBS to the detection of *C. jejuni* in South Africa, although our study strongly suggests that *Campylobacter* plays a significant role. Systematic studies with adequate clinical laboratory and epidemiological information including bacterial culture and serology would yield more conclusive results regarding the true picture of *Campylobacter*-induced AFP in South Africa.

Acknowledgements

This work constituted part of the first author's MSc. Med degree from the University of the Witwatersrand. We would also like to thank our colleagues at the Centre for Vaccines

and Immunology at the NICD for an efficient collaborative work.

Work was done at Centre for Enteric Diseases, National Institute for Communicable Diseases, a division of the National Health Laboratory Service, Johannesburg, South Africa

Financial support

The Global Disease Detection Centre through a Cooperative Agreement (5U19GH000571-02) with the National Health Laboratory Service financially supported this work. The contents of this manuscript are solely the responsibility of the authors and do not necessarily represent the official views of the Centers for Disease Control and Prevention.

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Corresponding author

Dr Anthony Marius Smith
Centre for Enteric Diseases, National Institute for Communicable Diseases, Private Bag X4, Sandringham, 2131, South Africa.
Phone: +27-11-5550348
Fax: +27-11-5550433
Email: anthonys@nicd.ac.za

Conflict of interests: No conflict of interests is declared.