ORIGINAL ARTICLE

Prospective study of pathogens in asymptomatic travellers and those with diarrhoea: aetiological agents revisited

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Abstract

Travellers' diarrhoea (TD) remains the most frequent health problem encountered by visitors to the (sub)tropics. Traditional stool culture identifies the pathogen in only 15% of cases. Exploiting PCR-based methods, we investigated TD pathogens with a focus on asymptomatic travellers and severity of symptoms. Pre- and post-travel stools of 382 travellers with no history of antibiotic use during travel were analysed with a multiplex quantitative PCR for *Salmonella*, *Yersinia*, *Campylobacter*, *Shigella*, *Vibrio cholerae* and five diarrhoeagenic *Escherichia coli*: enteroaggregative (EAEC), enteropathogenic (EPEC), enterotoxigenic (ETEC), enterohaemorrhagic (EHEC) and enteroinvasive (EIEC). The participants were categorized by presence/absence of TD during travel and on return, and by severity of symptoms. A pathogen was indentified in 61% of the asymptomatic travellers, 83% of those with resolved TD, and 83% of those with ongoing TD; 25%, 43% and 53% had multiple pathogens, respectively. EPEC, EAEC, ETEC and *Campylobacter* associated especially with ongoing TD symptoms. EAEC and EPEC proved more common than ETEC. To conclude, modern methodology challenges our perception of stool pathogens: all pathogens were common both in asymptomatic and symptomatic travellers. TD has a multibacterial nature, but diarrhoeal symptoms mostly associate with EAEC, EPEC, ETEC and *Campylobacter*.

Keywords: Aetiology, *Campylobacter*, diarrhoea, enteroaggregative *Escherichia coli*, enteropathogenic *E. coli*, enterotoxigenic *Escherichia coli*, quantitative RT-PCR, travel

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Introduction

Encountered by 10%–40% [1] of travellers visiting emerging economies, travellers' diarrhoea (TD) is the most common travel-associated health problem. Although approximately 80% of the cases with confirmed microbiological aetiology are ascribed to bacterial pathogens [1,2], numerous studies have, in lack of proper methodology, failed to recognize the responsible microorganism in up to half of the cases even when including viral and parasitic pathogens [1–4].

Traditionally, enterotoxigenic *Escherichia coli* (ETEC) has been considered the major causative pathogen for TD [1,2], but in recent research, enteroaggregative *E. coli* (EAEC) [5–8] and enteropathogenic *E. coli* (EPEC) [5,6,8,9] have been reported even more frequently. *Campylobacter* has been a common finding in travellers to South East Asia [1,2]. Viral pathogens, enterohaemorrhagic *E. coli* (EHEC) and enteroinvasive *E. coli* (EIEC), *Shigella* spp., *Salmonella* spp., and parasites have been detected less frequently [1,2]. The role of other pathogens such

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as non-cholera vibrios [10], *Arcobacter* [11] and enterotoxigenic *Bacteroides fragilis* [11] remains to be established.

The new methods provide far better coverage of the variety of aetiological agents than older approaches [5,7,12,13] and decrease the rate of unexplained TD cases to 5%–24% [6–8]. Furthermore, results obtained either by PCR [7–9,14] or culture-based methods [3,4,15] suggest that diarrhoeal pathogens are found even in asymptomatic travellers or travellers whose symptoms have already resolved [15]. However, in many studies the 'No TD' control group may have comprised, besides asymptomatic cases, also those with mild TD or participants whose TD symptoms had resolved before the time of sampling [3,4,7,9,14]. Even non-travellers have been used as controls [9,16]. Just a handful of investigations have applied strict criteria to the No TD control group [8,17,18].

To revisit the role of various TD pathogens, we explored separately travellers with ongoing disease, resolved disease and without any diarrhoeal symptoms during their entire journeys. The strict categorization is expected to provide accurate data on the frequency of symptomless carriage and so the actual significance of the various TD pathogens.

Material and methods

Study population

Participants were recruited prospectively at the Travel Clinic at Aava Medical Centre, Helsinki, Finland, between 1 March 2009 and 28 February 2010, among clients planning a journey outside the Nordic countries for a minimum of four nights. Of the initial 526 volunteers, 459 met the inclusion criteria, i.e. delivered (a) a pre-travel stool sample; (b) a completed pre-travel questionnaire; (c) a post-travel sample from the first (or second) stool after arrival; and (d) a completed post-travel questionnaire. Data on antimicrobial medication was available for 456/459 participants, 74 (16%) of whom reported having taken antibiotics during travel; the 382 with no antibiotic use (doxycycline as malaria prophylaxis not included) were included in the final analyses (Fig. 1).

The study protocol was approved by the Ethics Committee of the Department of Medicine, Helsinki University Hospital. Written informed consent was obtained from all participants.

We have earlier reported findings of resistant intestinal microbes in the same population [19], and included in our methodological study pilot data on diarrhoeal pathogens of 96 participants with TD [5].

Definition of travellers' diarrhoea and its severity

Travellers' diarrhoea was defined according to the WHO criteria [20] as the passage of three or more loose or liquid stools per day or, alternatively, more frequently than is normal for the individual.

Each traveller was classified in one of three categories: Asymptomatic (those having stayed free of any diarrhoeal symptoms during the entire journey), Resolved TD, and Ongoing TD.

Ongoing symptoms were characterized according to degree of severity: severe TD was defined as six or more diarrhoeal stools per day, TD accompanied by fever or haemorrhagic stools, or TD requiring hospitalization; mild TD involved one or two diarrhoeal stools per day; moderate TD comprised those with TD not fulfilling the criteria of severe or mild TD.

Stool collection

The stool samples were collected as swabs in Copan M40 Transystem tubes (Copan Diagnostics, Brescia, Italy) and mailed in special boxes, reaching the laboratory in 1–3 days. Once arrived, total nucleic acids were purified directly from stool swabs as described previously [5].

Identification of the stool pathogens

The analyses were carried out with a validated multiplex quantitative PCR method that was recently described [5] and thereafter applied to a group of 45 travellers visiting Benin together [8]. It covers the following pathogens: diarrhoeagenic *E. coli* including EPEC, ETEC, EAEC, EHEC and EIEC and *Shigella* as well as *Salmonella* spp., pathogenic *Yersinia enterocolitica*, *Yersinia pseudotuberculosis and Yersinia pestis*, *Vibrio cholerae* and *Campylobacter jejuni/coli*. The assay allows rapid and simultaneous examination of all these pathogens, providing results in just 4 h.

The questionnaires

The pre-travel questionnaire gathered information about the demographics and possible diarrhoeal symptoms at the time of first faecal sample. The post-travel questionnaire assessed diarrhoeal and other symptoms while abroad and immediately after returning home, in addition to which it covered countries visited and medication taken over the journey.

Statistical analysis

For categorical variables the statistical analyses were carried out with chi-squared tests, Fisher's exact test or binary logistic regression analysis when applicable. Statistical significance was defined as $p < 0.05$.The statistical analysis was conducted using SPSS 22 software (IBM Corp, Armonk, NY, USA).

Results

Demographics, travel itinerary and TD symptoms

Demographic and travel data are shown in Table 1 and details of TD symptoms are shown in Tables 2 and 3. In all, 65% of the

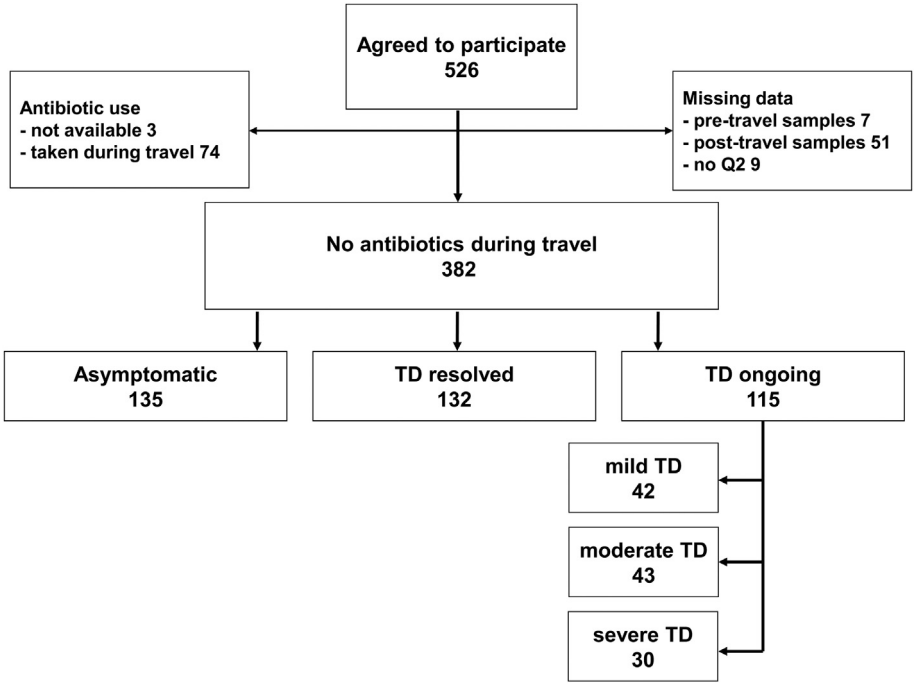

FIG. 1. Study protocol for investigating aetiology of travellers' diarrhoea with respect to occurrence and severity among Finnish travellers.

participants had TD: it was classified as severe in 22%, moderate in 34% and mild in 43%. At the time of post-travel stool sampling, 30% of the participants reported having ongoing diarrhoea, whereas the symptoms of 35% had already resolved.

Bacterial pathogens in stool samples

Findings in pre-travel stool samples. Of the pre-travel samples, 4% (14/382) were positive: 2% had EPEC, 1% had EAEC, 0.5% had ETEC and 0.3% had *Campylobacter*. One of these participants had mild diarrhoea at the time of sampling.

Findings in post-travel stool samples. The post-travel stool findings related to TD are presented in Table 2. A bacterial pathogen was detected in 75% of the samples. EPEC (46%) and EAEC (45%) proved the most common findings, followed by ETEC (20%), EHEC (9%), *Campylobacter* (7%), *Salmonella* (2%) and *Shigella*/EIEC (1%). Multiple pathogens were found in 40% of the post-travel samples.

Comparison of pathogen findings between the three categories: asymptomatic, resolved TD and ongoing TD. EPEC and ETEC proved more frequent among travellers with ongoing TD (55% and 37%, respectively; Table 2) than those with resolved disease (41% and 17%) and the asymptomatic group (45% and 9%). EAEC and *Campylobacter*, by contrast, were equally common among those with ongoing TD (51% and 8%) and resolved TD (57% and 12%), but less frequent in the asymptomatic group (28% and 1%). Multiple pathogens were detected in 25% of the samples of the asymptomatic participants, 43% in the group with resolved TD and 53% in the ongoing TD.

Findings in participants with mild TD proved similar to those of participants with moderate or severe ongoing disease (Table 3).

Discussion

Diarrhoea is the most common reason for returning travellers to contact healthcare [21,22]. Whereas the traditional laboratory diagnostics available in most clinical settings only cover a small number of diarrhoeal pathogens, the fast and accurate modern molecular methods, which allow diagnosis within hours instead of days, extend the coverage to the most common pathogens, i.e. diarrhoeagenic *E. coli* [5,12,13]. Our primary finding was that travellers carry various TD pathogens much more commonly than is generally believed: pathogens were detected in the stools of participants with resolved symptoms and even those remaining asymptomatic. Multiple pathogens proved common not only among participants with ongoing symptoms, but also the asymptomatic.

Findings in the asymptomatic group

One of our most impressive findings was that bacterial pathogens were detected in more than half (61%) of asymptomatic travellers, and multiple pathogens were found in almost half of these (42%). As this group only included those without any diarrhoeal symptoms during travel, the pathogens detected were not remains of bacteria having previously caused TD. A high proportion of pathogens has also been reported for asymptomatic travellers in a few earlier studies [3,7–9,14].

TABLE 1. Demographics of 382 Finnish travellers staying for at least 4 days outside Nordic countries and not taking antibiotics while travelling

	All *n* (%)	Asymptomatic *n* (%)	Resolved TD *n* (%)	Ongoing TD *n* (%)
Total	382	135 (35)	132 (35)	115 (30)
Sex				
Male	149 (39)	54 (40)	53 (37)	42 (40)
Female	233 (61)	81 (60)	79 (63)	73 (60)
Age (years)				
Age (mean)	40 (SD 17; range 0–77)	43 (SD 19; range 1–77)	36 (SD 17; range 0–76)	40 (SD 15; range 1–72)
Age group (years)				
0–17	31 (8)	16 (52)	11 (35)	4 (13)
18–30	105 (27)	18 (17)	45 (43)	42 (40)
31–50	130 (34)	45 (35)	46 (35)	39 (30)
51–64	83 (22)	38 (46)	20 (24)	25 (30)
over 65	33 (9)	18 (55)	10 (30)	5 (15)
Destination[1]				
South Asia	52 (14)	12 (23)	19 (36)	21 (40)
South East Asia	91 (24)	23 (25)	48 (53)	20 (22)
East Asia	5 (1)	3 (60)	0 (0)	2 (40)
Sub-Saharan Africa	171 (45)	61 (36)	53 (31)	57 (33)
North Africa and Middle East	11 (3)	7 (64)	1 (9)	3 (27)
Latin America	36 (9)	16 (44)	11 (31)	9 (25)
Europe, North America and Australia	16 (4)	13 (81)	0 (0)	3 (19)
Duration of travel in days, mean (information missing 5)	21 (median 16, SD 17 range 5–146)	16 (median 15, SD 10 range 5–81)	29 (median 19, SD 24 range 5–146)	18 (median 15, SD 10, range 5–60)
7 days or less	11 (3)	8 (6)	1 (1)	2 (2)
8–14 days	111 (29)	52 (39)	18 (14)	41 (37)
15–30 days	196 (52)	64 (47)	74 (57)	58 (52)
31 days or more	59 (16)	11 (8)	37 (29)	11 (10)
Chronic underlying illness[2]	78 (20)	40 (30)	22 (17)	16 (14)

The data are given separately for those with no TD during journey, those with resolved, and those with ongoing TD.
[1]Travel destinations were grouped into regions as described earlier [22].
[2]Asthma, cardiovascular disease, diabetes, coeliac disease, inflammatory bowel disease, epilepsy, rheumatic disease, malignancy during the past 10 years.

Consistent with some previous investigations [3,7,14,15,18]. EAEC and EPEC were the two most common pathogens among the asymptomatic travellers, whereas, contrary to findings elsewhere [3,7], *Campylobacter* and ETEC proved rare. This may have resulted from our rigorous inclusion criteria for the group, whereas other studies may have included travellers with resolved or mild symptoms. All these data emphasize the importance of having an asymptomatic control group consisting of travellers with no symptoms during their journeys, when assessing the clinical significance of the various stool pathogens.

Findings in TD groups

The pathogen findings for travellers with TD differed significantly from those for the asymptomatic, whereas there was no difference among those with ongoing TD, i.e. between participants with mild, moderate or severe diarrhoea. In TD, EAEC and EPEC proved more prevalent than ETEC, which has traditionally been considered the central TD pathogen [1,2]. In recent research [6–8], EAEC and EPEC have been detected more frequently than ETEC, probably because of deploying advanced methods that afford better coverage of the pathogens. We found an association between diarrhoeal symptoms and EPEC, EAEC, ETEC and *Campylobacter*. A comparison between participants with ongoing symptoms and those with resolved TD suggests that ETEC and EPEC disappear from the stools after resolution of symptoms, whereas EAEC and *Campylobacter* persist for longer.

We found EAEC more frequently in the stools of travellers with ongoing or resolved symptoms than the asymptomatic group, confirming earlier reports of EAEC's role as a TD pathogen [7,23,24]. However, as EAEC is common also among the asymptomatic, its significance in individual cases should be viewed with caution.

This is one of the first studies enabling comparison between EPEC findings in post-travel stools of symptomatic and asymptomatic travellers. Until now, despite being recognized as a major pathogen in childhood diarrhoea in developing countries [25], EPEC's role in TD has been disputable: its frequency has been similar among those with and without TD [3,8,14,17], travel histories of controls have not been provided [16], not all have been travellers [9], or a control group has been lacking altogether [6]. We detected EPEC more frequently in ongoing TD than among asymptomatic participants or those with symptoms resolved, which suggests a role for this pathogen in adult TD. Indeed, these results and lack of earlier data on EPEC [1,2] point to a need for more research into its role in TD.

In many earlier TD studies [2], multiple pathogen findings have been rare. In our data, half (53%) of those with ongoing TD symptoms and every fourth (25%) asymptomatic traveller had two or more pathogens. This accords with a few other recent

TABLE 2. Bacterial findings in 382 returning travellers having experienced travellers' diarrhoea as defined by current WHO criteria[1]

		Ongoing TD at time of sampling	No TD at time of sampling		Univariate statistics					
	Total	Ongoing TD	Asymptomatic	Resolved TD	Ongoing TD versus asymptomatic		Ongoing TD versus resolved TD		Resolved TD versus asymptomatic	
	n (%)	*n* (%)	*n* (%)	*n* (%)	OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p
Total:	382	115 (30)	135 (35)	132 (35)						
Any bacterial pathogen	287 (75)	96 (83)	82 (61)	109 (83)	3.3 (1.8–6.0)	<0.001	0.9 (0.5–1.8)	0.851	3.1 (1.7–5.4)	<0.001
Two or more pathogens	152 (40)	61 (53)	34 (25)	57 (43)	2.5 (1.3–4.5)*	0.004	1.6 (0.9–2.8)*	0.105	1.5 (0.9–2.6)*	0.139
Diarrhoeagenic *E. coli* (DEC)	277 (73)	95 (83)	81 (60)	101 (77)	2.2 (1.3–3.7)	0.004	1.5 (0.8–2.7)	0.239	3.2 (1.8–5.7)	<0.001
EPEC	174 (46)	63 (55)	57 (42)	54 (41)	1.7 (1.0–2.7)	0.048	1.6 (1.1–2.9)	0.030	0.9 (0.6–1.5)	0.828
EAEC	171 (45)	58 (50)	38 (28)	75 (57)	2.6 (1.5–4.4)	<0.001	0.7 (0.5–1.3)	0.316	3.4 (2.0–5.6)	<0.001
ETEC	76 (20)	42 (37)	12 (9)	22 (17)	5.9 (2.9–11.9)	<0.001	2.9 (1.6–5.2)	<0.001	2.1 (1.0–4.3)	0.060
EHEC	34 (9)	7 (6)	10 (7)	17 (13)	0.8 (0.3–2.2)	0.680	0.4 (0.2–1.1)	0.079	1.8 (0.8–4.2)	0.143
Non-DEC	36 (9)	12 (10)	4 (3)	20 (15)	3.8 (1.2–12.2)	0.024	0.7 (0.3–1.4)	0.273	5.8 (1.9–17.6)	0.002
Campylobacter	26 (7)	9 (8)	1 (1)	16 (12)	11.4 (1.4–91.2)	0.022	0.6 (0.3–1.5)	0.268	18.5 (2.4–141.5)	0.005
Salmonella	29 (2)	3 (3)	3 (2)	3 (2)	1.2 (0.2–6.0)	0.842	1.1 (0.2–5.8)	0.864	1.0 (0.2–5.2)	0.978
EIEC/*Shigella*	3 (1)	1 (1)	0 (0)	2 (2)	N/A	0.996	0.6 (0.1–6.4)	0.648	N/A	0.996
Vibrio cholerae	0 (0)	0 (0)	0 (0)	0 (0)	N/A	N/A	N/A	N/A	N/A	N/A
Yersinia spp.	0 (0)	0 (0)	0 (0)	0 (0)	N/A	N/A	N/A	N/A	N/A	N/A

Results are presented in relation to symptoms during travel and at the time of post-travel stool sampling. The following bacterial pathogens were explored: enteropathogenic (EPEC), enteroaggregative (EAEC), enterotoxigenic (ETEC), enterohaemorrhagic (EHEC) and enterionvasive (EIEC) *Escherichia coli* or *Shigella*, as well as *Salmonella* spp., *Yersinia* spp., *Vibrio cholerae* and *Campylobacter coli/jejuni*. Pathogens were found in all groups: Ongoing TD, Resolved TD and Asymptomatic, frequency decreasing respectively.
*Compared with one pathogen.
[1]WHO definition: passage of three or more loose or liquid stools per day or, alternatively, more frequently than is normal for the individual [20].

investigations reporting 18%–79% of participants co-infected [3,7–9,11,14]. Importantly, in many earlier studies [2] diarrhoeagenic *E. coli* have not been comprehensively explored. Prospective research using modern molecular methods is needed to pinpoint the actual involvement of each pathogen.

Considerations of the PCR method

While up to 50% of cases have remained unexplained in aetiological investigations of TD [1–4], the PCR-based method used herein revealed a pathogen in 83%, concurring with recent studies employing these techniques [6–8]. In addition to providing a broader coverage of stool pathogens, PCR methods are more sensitive than stool cultures. The present method has been validated with culture confirmation as described earlier [5].

Our finding of multiple pathogens in 40% of all TD stools attests to the significance of the methodology used: the role of each pathogen can only be evaluated if data on potential other diarrhoeal microbes are available. Although a broader coverage of the range of pathogens will certainly enhance the accuracy of future studies, it will also entail new challenges to interpreting results, for example in cases with multiple pathogens.

Limitations

Three limitations deserve to be discussed. First, the participants provided the second stool sample only after travel and, therefore, the symptoms may already have resolved and some pathogens disappeared, whereas others not initially causing the symptoms may still be present. To tackle this problem, we made separate comparisons between the groups with ongoing and resolved symptoms (results discussed above). Our results may nonetheless underestimate the proportion of certain pathogens, especially ETEC, which has been suggested to disappear rapidly from the stools [15,26]. The second limitation, the inablility of quantitative

TABLE 3. Relation between findings of EPEC, EAEC, ETEC, *Campylobacter* and severity of ongoing TD symptoms at sampling time

		No TD	Ongoing TD symptoms			Univariate statistics
	Total	Asymptomatic	Mild TD	Moderate TD	Severe TD	
	n (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	Severity of ongoing TD symptoms, p-value
Total	250	135 (50)	42 (17)	43 (17)	30 (12)	
Any bacterial pathogen	96 (71)	82 (61)	35 (83)	37 (86)	24 (80)	0.791
EPEC	120 (48)	57 (42)	22 (52)	28 (65)	13 (43)	0.170
EAEC	96 (38)	38 (28)	19 (45)	26 (60)	13 (43)	0.248
ETEC	54 (22)	12 (9)	11 (26)	19 (44)	12 (40)	0.204
Campylobacter	10 (4)	1 (1)	3 (7)	3 (7)	3 (10)	0.875

Data given on those remaining asymptomatic during travel and those with ongoing TD symptoms at time of sampling (n = 250).
EPEC, enteropathogenic *Escherichia coli*; EAEC, enteroaggregative *E. coli*; ETEC, enterotoxigenic *E. coli*; TD, tavellers' diarrhoea.

PCR methods to distinguish between live and dead bacteria, actually constitutes a shortcoming in all studies employing such assays. On the other hand, free DNA is unlikely to resist active DNAases during its entire passage through the gastrointestinal tract [27,28]. Third, we did not cover parasitic, viral and some other potential bacterial pathogens. Analysing them might further diminish the proportion of unexplained TD cases.

Clinical considerations

Adopting the novel advanced methodology into routine use, clinicians have new challenges to meet: when to take the test and how to interpret the results. We instruct our healthcare professionals that tests are warranted only in instances where microbiological diagnostics would earlier have been used. Evaluating the significance of the various pathogens detected is complicated by the great proportion of multiple bacterial findings (53% of cases with ongoing symptoms). The most common pathogens, diarrhoeagenic *E. coli*, usually do not require antibiotic treatment. The same applies to most TD cases: the great majority (up to 78%) remain mild or moderate and resolve spontaneously [8,29,30]. Indeed, caution with antibiotics is also highlighted in our recent study showing that antibiotics taken against TD predispose travellers to colonization with multiresistant intestinal bacteria [22].

Conclusion

PCR-based methodology changes our perception of stool pathogens, because all pathogens are seen in travellers, asymptomatic and symptomatic alike, both showing high rates of detection. In light of our analysis, it appears vital that future studies include an asymptomatic control group. Our results support the current view that TD only rarely requires antibiotic treatment. The data discourage prescribing antimicrobials merely on the basis of microbiological findings, for some of the pathogens detected may not have caused any symptoms. TD has a multibacterial nature, yet diarrhoeal symptoms are mostly associated with EAEC, EPEC, ETEC and *Campylobacter*.

Transparency declaration

All authors declare no conflicts of interest.

Authors' contributions

Study concept and design—JA, JK, AK; drafting of manuscript—TL, AK; statistical analysis—TL. All authors contributed to acquisition of data analysis and interpretation of results and they all gave final approval of the version published.

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