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Original Article

Original Article

TaqMan Array Card testing of participant-collected stool smears to determine the pathogen-specific epidemiology of travellers' diarrhoea†

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Abstract

Background: We assessed the compliance with self-collection of stool smears on Whatman® FTA® Elute Card (FTA Card) and detection of travellers' diarrhoea (TD)-associated pathogens by using a quantitative Polymerase Chain Reaction (PCR) assay [customized TaqMan® array card (TAC)] in a prospective, observational cohort of travellers.

Methods: Enrolled travellers documented symptoms on a travel diary and collected an FTA Card during a diarrhoeal episode, or at the end of travel if they remained asymptomatic. TAC testing was performed on FTA Cards from TD cases and 1:1 matched asymptomatic controls and 1:1 matched loose stool cases that did not meet TD criteria. Odds ratios were used to determine the association between detected pathogens and TD.

Results: Of 2456 travellers, 484 (19.7%) completed an illness diary and met TD criteria, and 257 (53.1%) collected an FTA Card during the TD episode. FTA Cards were stored for a median of 2 years at room temperature (IQR: 1–4 years) before extraction and testing. The overall TAC detection rate in TD cases was 58.8% (95% CI: 52.5–64.8). Enterotoxigenic *Escherichia coli* was the most common pathogen in TD cases (26.8%), and 3.5% of samples were positive for norovirus. The odds of detecting TD-associated pathogens in 231 matched cases and asymptomatic controls were 5.4 (95% CI: 3.6–8.1) and 2.0 (95% CI: 1.1–3.7) in 121 matched TD and loose stool cases ($P < 0.05$). Enteroaggregative *E. coli* was the most common pathogen detected in asymptomatic controls and loose stool cases. Detection of diarrhoeagenic *E. coli*, Shigella/enteroinvasive *E. coli* and *Campylobacter* spp. was significantly associated with TD.

Conclusion: FTA Cards are a useful adjunct to traditional stool collection methods for evaluating the pathogen-specific epidemiology of TD in austere environments. Qualitative detection of pathogens was associated with TD.

Measures to improve compliance and quality of FTA Card collection with decreased storage duration may further optimize detection.

Key words: Travellers' diarrhoea, PCR, TaqMan Array Card, Whatman FTA Elute, enteropathogen

Introduction

Travellers' diarrhoea (TD) is common during military deployment and travel to high-TD-risk regions, with a pooled incidence of >30 cases per 100 person-months.[1] Evaluating the pathogen-specific epidemiology of TD is important for vaccine development and optimizing treatment but is hindered by the challenge of collecting stool specimens and clinical data during travel. Thus, PCR-based studies evaluating the association between pathogen detection and clinical disease are limited to single-centre efforts that collect stool samples prior to and following travel or from medically attended TD cases, and they are impacted by sampling bias.[1–6] Unsupervised, participant collection of biospecimens and completion of symptom-based questionnaires have been widely adapted for Coronavirus Disease 2019 and influenza-like-illness surveillance,[7,8] and such approaches are greatly needed for TD surveillance in order to reduce study budgets, increase the sample size and improve the convenience of study participation. Studies evaluating filter-paper-based matrices for storage and shipment of diarrhoeal smears utilize laboratory staff for the preparation of smears.[9–12]

The Whatman® FTA® Elute card (FTA Card, GE Healthcare Life Sciences, Marlborough, MA, USA) is able to lyse cells, bind PCR inhibitors and store nucleic acid at room temperature for prolonged periods, thus facilitating the collection and transportation of faecal samples to testing sites. We evaluated the compliance with self-collection of symptom data and stool smears on FTA Card (FTA Card) in a prospective, multicentre cohort study of US Department of Defense (DoD) beneficiaries travelling or deploying outside the continental US for ≤ 6.5 months (TravMil). FTA Cards were tested using a quantitative PCR [TaqMan® array card (TAC), Life Technologies, Carlsbad, CA] to evaluate the pathogen-specific TD epidemiology and to determine the odds of detecting TD-associated pathogens using a nested case–control design.

Methods

The TravMil study is approved by the Uniformed Services University Institutional Review Board (Bethesda, MD). The study cohort primarily consisted of deployed active duty personnel (42.6%), personnel on vacation travel (20.1%), humanitarian work (8.2%) and other smaller groups (Table 1). Consenting adults were enrolled pre-travel at eight US military travel clinics (seven in the USA and one in Landstuhl, Germany), and travel-related prescriptions, demographics and itineraries were abstracted. Travellers with itineraries limited to Western or Northern Europe, Canada or New Zealand were excluded due to the low TD risk. Participants documented the number of loose or liquid stools per 6-h period during a diarrhoeal episode, associated symptoms, treatment and impact on daily activities in a travel illness diary. Self-collection of FTA Cards was an optional study procedure between January 2010 and July 2014 and became a required procedure between August 2014 and September 2018. FTA Cards, pre-labelled with subject identification codes and enclosed in pre-addressed, pre-stamped envelopes were provided, and subjects were instructed to collect an FTA Card on 2 consecutive days during a diarrhoeal episode or within 2 days prior to return or 7 days after return if they remained asymptomatic. Following a bowel movement, subjects smeared the soiled toilet paper across the FTA Card, closed the flap and wrote the date of collection and whether they had diarrhoea at the time of specimen collection. FTA Cards were stored in a multi-barrier pouch at room temperature and mailed to the central testing location at Naval Medical Center Portsmouth, VA. The current analysis included adults enrolled between January 2010 and September 2018.

Definitions

The travel illness diary was used to determine whether participants met the criteria for TD, defined as ≥3 unformed stools in a 24-h period or 2 unformed stools with ≥1 accompanying symptom (nausea, vomiting, fever, blood in stool and abdominal pain) in a 24-h period. TD cases were further divided into mild acute watery diarrhoea (AWD) (i.e. AWD with no impact on daily activities), moderate/severe AWD (AWD with impact on daily activities) and invasive TD (fever or blood in stool). Subjects who experienced loose or liquid stool but did not meet TD criteria were classified as loose stool cases.[13] Subjects who did not report any loose or liquid stool on the diary were classified as asymptomatic. Categories were mutually exclusive, and only the first episode of TD or loose stool per subject trip was included in the analysis.

Analytic plan

We evaluated subject compliance with the travel illness diary and FTA Cards, timing of FTA Card collection relative to the onset of the diarrhoea and specimen arrival at the site. TD cases that collected an FTA Card within 5 days prior to start of a TD episode or up to 5 days after the end of the episode were selected for TAC testing. One FTA Card per diarrhoeal episode was tested. Two nested case–control analyses were performed to determine the association between pathogen detection and TD. TD cases who collected an FTA Card during the pre-specified period were matched 1:1 with (i) asymptomatic travellers who collected an FTA Card during the pre-specified period and (ii) loose stool cases that collected an FTA Card within 5 days prior to start of an episode or up to 5 days after the end of the episode. Controls (i.e. asymptomatic travellers and loose stool cases) were matched without replacement based on five criteria: (i) age (18–50 years or >50 years), (ii) duration of travel (≤6 weeks

Table 1. Demographic characteristics of 231 matched TD cases and asymptomatic controls

	TD cases (%)	Controls (%)
Age—years		
18–29	52(22.5)	49(21.2)
30–50	105(45.4)	108(46.8)
>50	74(32.0)	74(32.0)
Median—years (IQR)	41(30–56)	42(30–54)
Male gender	132(57.1)	154(66.7)
Active duty service members on deployment	104(45.0)	103(44.6)
Regions of travel		
Caribbean	11(4.8)	11(4.8)
Central America	31(13.4)	31(13.4)
East and North Asia	14(6.1)	14(6.1)
Europe	2(<1)	2(<1)
South America	22(9.5)	22(9.5)
Sub-Saharan Africa	88(38.1)	88(38.1)
South-East Asia	63(27.3)	63(27.3)
Duration of travel		
<2 weeks	67(29.0)	91(39.4)
2–3 weeks	102(44.2)	86(37.2)
4–5 weeks	25(10.8)	15(6.5)
≥6 weeks	37(16.0)	39(16.9)
Median (IQR): days	17(13–28)	16(10–26)
Cruise ship travel	6(2.6)	6(2.6)
Reason/purpose for travel		
Military	90(39.0)	107(46.3)
Vacation	53(22.9)	40(17.3)
Business	2(<1)	4(1.7)
Visiting friends/family	10(4.3)	6(2.6)
Teaching/study	3(1.3)	5(2.2)
Providing medical support	2(<1)	2(<1)
Humanitarian work	20(8.7)	18(7.8)
Multi-purpose travel	48(20.8)	47(20.3)
TD severity		
Mild AWD	101(43.7)	
Moderate–severe AWD	65(28.1)	
Invasive TD	65(28.1)	
Antibiotic self-treatment	58(25)	
Self-treatment with anti-motility agent	65(28)	
Timing of antibiotic and stool card collection		
1 day prior to stool card collection	15(25.9)	
Same day	27(46.6)	
After stool card collection	16(27.6)	
Duration of diarrhoea		
≤24 h	90(39.0)	
2 days	87(37.7)	
>2 days	54(23.4)	

or >6 weeks), (iii) type of travel (cruise ship travel vs. no cruise ship travel), (iv) region of travel (South-East Asia, South America, Sub-Saharan and North Africa, Central America, Caribbean, Europe and East and North Asia) and (v) duration between sample collection and extraction by using a stratified approach, that is, TD FTA cards were matched with asymptomatic or loose stool FTA Cards that had a storage duration of +/−3 months of the storage duration for the TD cases. For example, a TD FTA Card with a 24-month storage duration was matched to an asymptomatic FTA Card stored between 21 and 27 months. If no suitable controls were found, the storage duration was expanded to +/−12 months of the storage duration for the TD case; any remaining TD cases were matched to controls regardless of the storage duration.

Extraction and PCR

TAC is a 384-well singleplex, quantitative, real-time PCR format that allows for an estimation of nucleic acid quantity by the quantification cycle (Cq). A customized TAC panel was developed for detection of 20 TD pathogens and associated virulence factors (Supplementary Table S1 available as Supplementary data at *JTM* online).

Between January 2010 and August 2016, FTA Cards were maintained at room temperature until extraction and TAC testing. In August 2016, we began storing all FTA Cards at −20°C to reduce genomic degradation. Three 3-mm discs were punched from the FTA Card sample area and were pulse-vortexed with 500 μl of sterile water. The discs were mixed with 100 μl of sterile water and two extrinsic controls (bacteriophage MS2 and phocine herpesvirus), subjected to bead beating and incubated at 95°C for 5 min.[14–17] Cases and controls were tested simultaneously with extraction blanks and with a Cq value of 35 as the positive cut-off.[14,16] Positive results were considered valid only when the corresponding extraction blank was negative for the relevant target; negative results were considered valid only when the external controls were positive for the sample.

Statistical analysis

Pearson's χ^2 test or Fisher's exact test were performed for univariate analysis of categorical variables, and Mann–Whitney *U* was performed for continuous variables. When examining the association between pathogen detection and TD cases and controls, a multivariable logistic regression adjusted with the storage duration was applied to estimate the odds ratio (OR) and 95% CI. Statistical analysis was conducted using SAS version 9.4 (SAS Institute, Cary, NC).

Results

Three thousand six hundred and eighty adults were enrolled between 2010 and 2018. The median duration of travel was 21 days (IQR: 13–46 days). Sixty-seven percent ($n = 2456$) recorded symptoms on the travel illness diary, and of these, 19.7% ($n = 484$) met TD criteria [incidence rate: 3.48 cases/100 person weeks of travel; mild AWD: 205 cases (42.4%), moderate or severe AWD: 170 cases (35.1%); febrile diarrhoea or dysentery: 109 cases (22.5%)] (Figure 1). Severity of AWD could not be determined for one subject with missing data. The median duration of TD was 2 days (IQR: 1–3 days), and the median interval between departure and TD onset was 12 days (IQR: 5–37 days). Among subjects who completed a diary, compliance with FTA Card collection was higher in subjects who were symptomatic [loose stool: 62.7% (158/252), TD: 62.6% (303/484)] vs. asymptomatic (50.7%, 872/1720). The majority

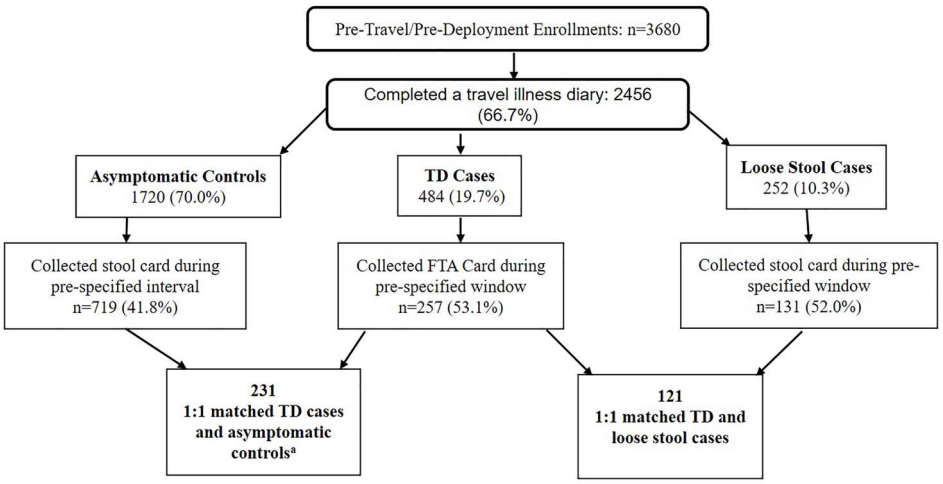

Figure 1. Flow diagram of the paired case and control subjects; patients excluded from match: TD cases (failed DNA/RNA controls = 2; failed RNA controls = 3; travelled to >1 region = 4; unable to determine which region TD developed = 1; matching control sample was excluded = 7); controls (failed DNA/RNA controls = 3; failed RNA controls = 5; matching case sample was excluded = 3).

of TD cases and asymptomatic controls collected FTA Cards within the pre-specified period (Supplementary Figures S1 and S2 available as Supplementary data at *JTM* online). The median duration between sample collection and receipt at the site was 10 days (IQR: 5–21 days; range: 0–232 days) and was expectedly shorter in subjects who remained asymptomatic and collected the sample at the end of travel [7 days (IQR: 3–15 days; range: 0–219 days)] vs. those who developed loose stool (18 days IQR: 10–33 days; range: 0–232 days) or met TD criteria 20 days (IQR: 12–36 days; range: 0–194 days). FTA Cards were stored for a median of 2 years at room temperature (IQR: 1–4 years; range: 0–8) before extraction and testing due to delays in procuring TAC and matching cases and controls for batch testing.

Two hundred and fifty-seven TD cases collected an FTA Card during the pre-specified collection window (Figure 1 and Supplementary Figure S1 available as Supplementary data at *JTM* online): 84.0% had diarrhoea at the time of collection, 2.7% collected an FTA card within 5 days prior to start of the TD episode and 10% collected an FTA within 5 days after TD episode onset but were not symptomatic at the time of collection. The overall TAC detection rate was 58.8% (95% CI: 52.5–64.8) and was not significantly different in cases of mild AWD (52.2%), moderate/severe AWD (61.6%) and invasive TD (66.7%; $P = 0.13$) (Figure 2). Enterotoxigenic *Escherichia coli* (ETEC) (26.8%; $n = 69$) was the most common pathogen: 21 (30.4%) were positive for heat-labile toxin (LT) alone, 31 (44.9%) were positive for heat-stable toxin (ST) alone, 17 (24.6%) were positive for both LT and ST and 35 (50.7%) were positive for a major colonization factor [Colonization Factor Antigen I or Coli Surface (CS) antigens CS1-CS6]. Twenty-seven TD cases were LT-/ST-negative but were positive for a colonization factor. Diarrhoeagenic *E. coli* [i.e. ETEC, enteropathogenic *E. coli* (EPEC), or enteroaggregative *E. coli* (EAEC)] frequently occurred as co-pathogens (e.g. 47 of the 64 co-pathogen detections were ≥1 diarrhoeagenic *E. coli*). *Campylobacter* spp. were most common in Asia (16.3%) and in cases of invasive TD ($n = 18.8\%$). Only 3.5% of TD samples were positive for Norovirus.

TAC detection in TD cases vs. asymptomatic controls

Two hundred and thirty-one TD cases were matched 1:1 with controls (Table 1 and Figures 1 and 3). The median duration of FTA Card storage was 2.3 years (IQR: 1.2–3.6) for TD cases and 2.6 years (IQR: 1.6–3.9) for controls (Figure 4): 152 matched controls had a storage duration within 3 months of TD cases; 61 matched controls had a storage duration within 1 year of TD case and 18 pairs were matched without regard to storage duration. The odds of detecting a TD-associated pathogen was 5.4 (95% CI: 3.6–8.1, P value < 0.0001; detection rate 59.7% in TD cases vs. 21.2% in controls) and the association with TD was observed across pathogens and multi-pathogen detection (Figure 5). Among the TD cases, pathogen detection was numerically higher in invasive TD [68.3% (43/63)] and moderate or severe AWD cases [61.3% (38/62)] than mild AWD [53.8% (57/106)]. Major colonization factors were present in 58% (36/62) of ETEC TD cases and 28.6% (2/7) asymptomatic controls with ETEC. EAEC was the most common pathogen in controls (14.7%; $n = 34$), and 75% of EAEC detections in TD cases were samples with multi-pathogens. Stratification by Cq values did not improve the association with TD compared to qualitative detection, although sample size limitations precluded stratification and resulted in large confidence intervals (Supplementary Table S2 available as Supplementary data at *JTM* online).

TAC detection in TD vs. loose stool cases

FTA Cards from 121 loose stool and TD cases were matched (39 mild TD, 76 moderate or severe AWD and 37 invasive TD). The odds of detecting TD associated pathogens was lower when compared to loose stool cases [OR: 2.0 (95% CI: 1.1–3.7), P value = 0.029; detection rate TD: 55.4% (67/121) vs. loose stool: 33.1% (40/121)] (Supplementary Figure S3 available as Supplementary data at *JTM* online). Detection of ETEC, EPEC, *Campylobacter* and multi-pathogen detection was significantly

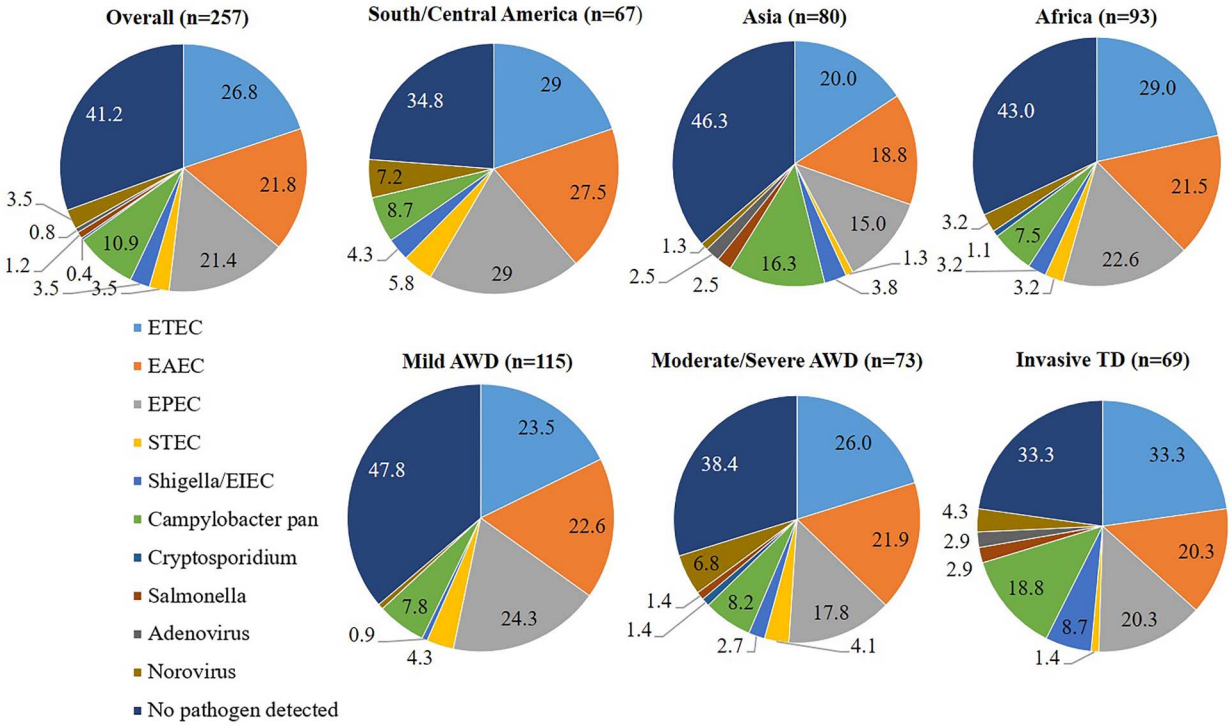

Figure 2. Distribution of pathogen among TD cases who collected an FTA card during the pre-specified period ($n = 257$); subjects counted more than once in pie-chart for co-pathogen detections; co-pathogen detections—overall: 64 (24.9%), Central/South America: 21(30.4%), Asia: 15 (18.8%), Africa: 24 (25.8%), mild AWD: 19 (25.3%), moderate/severe AWD: 28 (25.2%) and invasive TD: 17 (23.9%).

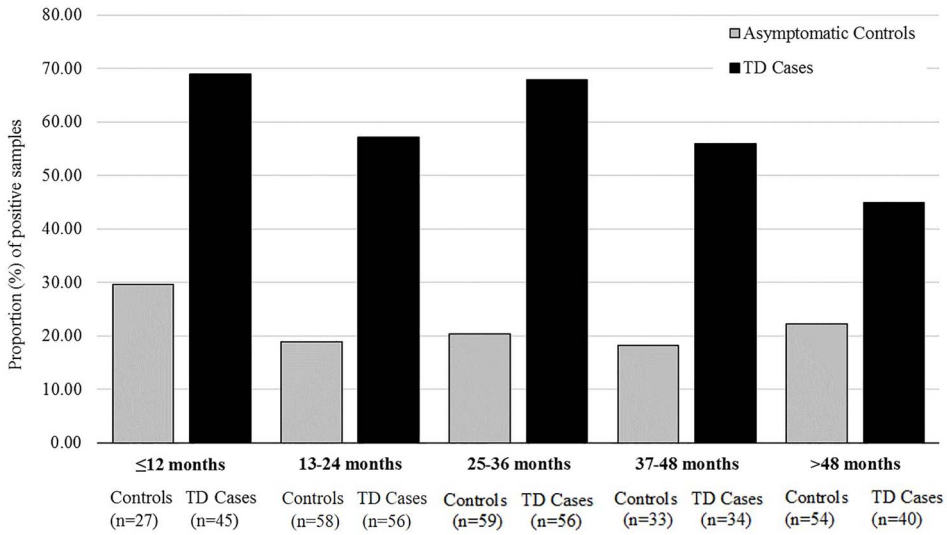

Figure 3. Proportion of the 231 matched TD cases and asymptomatic controls with a pathogen detected, stratified by the duration between sample collection and extraction/testing.

associated with TD. There was no significant difference in the mean Cq values of TD and loose stool cases across pathogens, although the analysis was limited by the small sample size.

Discussion

Our results support the use of participant-collected FTA Cards and symptom data as an adjunct to traditional methods for evaluating the pathogen-specific epidemiology of TD in austere environments and offer important insights regarding the expected compliance, detection rates and measures for further optimization. A pathogen was detected in 58.8% (95% CI: 52.5–64.8) of TD cases, which is in the range of detection estimates from clinic-based studies using stool culture or molecular testing. The Global Travelers' Diarrhea Study reported a detection rate of 59% by quantitative PCR and culture-based methods,[18] while other cohorts have reported detection rates in the range of 62–89%.[1,2,5,19] The wide variability across studies can be related to

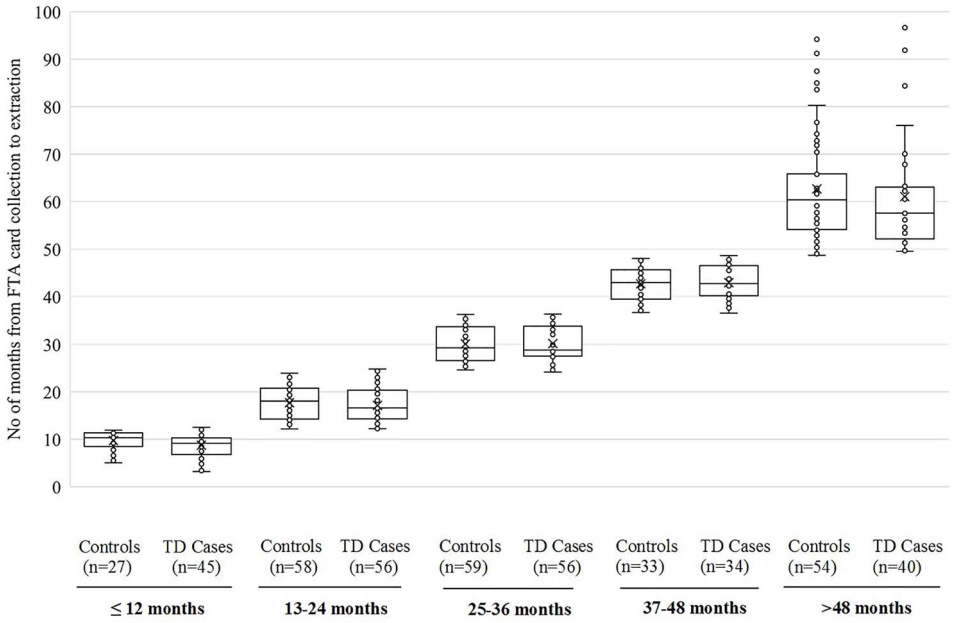

Figure 4. Duration between FTA Card collection and extraction for 231 matched TD cases and asymptomatic controls.

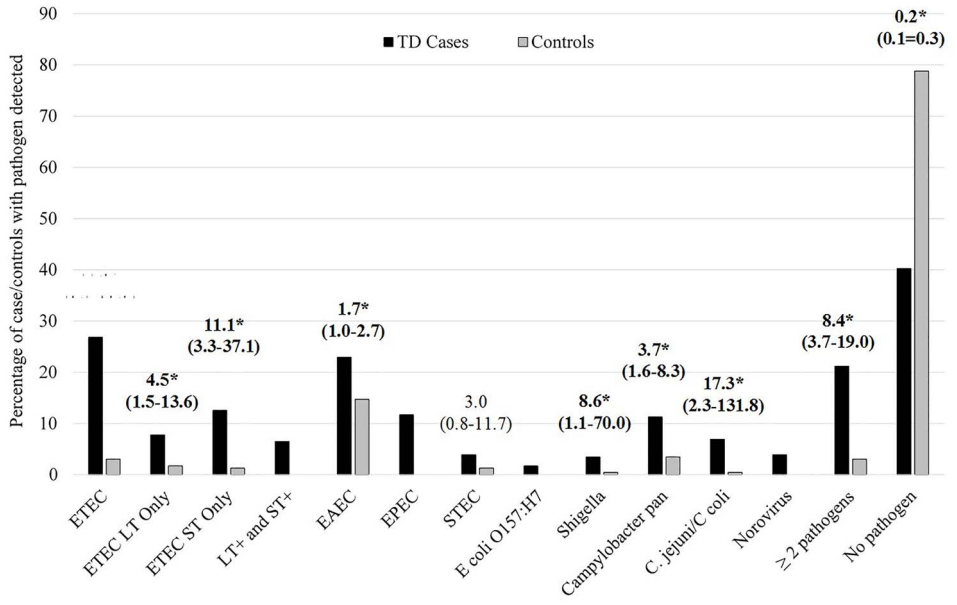

Figure 5. Distribution of pathogens and ORs in 231 matched TD cases and asymptomatic controls; pathogens with <2 positive samples not shown in bar-graph: *Aeromonas* (TD cases: 0; controls: 1); *Clostridium difficile* (TD cases: 0; controls: 1); *Cryptosporidium* (TD cases: 1; controls: 0); Adenovirus 40/41 (TD cases: 1; controls: 0), Rotavirus (TD cases: 1; controls: 0); no samples were positive for *Vibrio. parahemolyticus*, *Giardia*, *Entamoeba histolytica*, *Cyclospora*, Astrovirus and Sapovirus; significant OR (adjusted for storage duration) with $P < 0.05$ in bold and with asterisk.

several factors, including differences in traveller characteristics (e.g. purpose of travel and nationality),[18,20] region of travel [e.g. lower detection rates reported in Africa and Latin America (50%) vs. South-East Asia (80%)],[1,18] TD severity and antibiotic treatment.[6,16,20,21] Although FTA Card compliance in TD cases was low (62%), those who collected an FTA Card did so during the symptomatic period and either did not take antibiotics or collected an FTA Card prior to or within 1 day of starting antibiotics. Despite these measures, TAC detection was lower than estimates in two prior studies using TAC on stool [85.6% (95% CI: 79.4–90.4%) and 78.6% (95% CI: 72.0–84.3%) respectively] and TAC on FTA Cards prepared by laboratory personnel [73.2% (95% CI: 66.3–79.5%)], suggesting that the lower detection rates in participant-collected FTA Cards could be due to inadequate stool sampling and/or genomic degradation with prolonged storage at room temperature. Determining the adequacy of participant-collected stool smears for assessing total bacterial/viral content is challenging due to differences in mass between solid and watery stool and varying PCR inhibition and efficiency.[14] We included FTA Cards for TAC testing, which were

collected 5 days prior to the first unformed stool associated with a TD episode. This a priori criterion was added to capture samples collected within the incubation period of TD pathogens, especially among TD cases who may have collected FTA Cards while experiencing loose stools prior to meeting TD criteria. Only 2% (7/303) of TD samples were collected within this window (Supplementary Figure S1 available as Supplementary data at *JTM* online), six of which were collected within 2 days prior to the first unformed stool associated with a TD episode. Based on these findings, we recommend a window of within 2 days prior to the first unformed stool associated with a TD episode. Eighty-four percent of TD FTA Cards were stored at room temperature for 1–6 years, and higher TAC detection rates were observed in FTA Cards tested within 12 months of collection (68.9%) vs. those stored for over 4 years (45%). We were unable to evaluate the impact of storage temperature on detection since only 37 TD and 74 control FTA Cards were collected after we started storing cards at −20°C. We plan to store future FTA Cards at −20°C and extract them within 3 months of receipt.

The regional distribution of pathogens was similar to prior reports, with some notable differences. Diarrhoeagenic *E. coli* were the most common pathogens detected across geographic regions, but unlike other cohorts reporting a predominance of ETEC detection,[1] the proportions of EAEC and EPEC were almost equivalent to ETEC and frequently occurred as co-pathogens. In addition, the proportion of EAEC in Africa and *Campylobacter* and *Salmonella* in Asia was lower than prior estimates.[1,3,18–20] Norovirus detection was significantly lower in our cohort (3.5%) compared to prior estimates (12–24%)[3,18,20] due to RNA degradation with prolonged storage at room temperature.[22] Giardia detection was also lower than reported in other cohorts since adventure travellers are rarely seen in military travel clinics and active duty personnel and vacation travellers have access to potable water, reducing the risk of giardiasis. In addition, genomic degradation due to prolonged storage could have impacted detection. Compared to matched asymptomatic controls, the odds of detecting TD-associated pathogens in cases were 5.4 (95% CI: 3.6–8.1). A lower OR was observed when comparing TD to loose stool cases. Bodhidatta *et al*. reported a similar OR of 2.86 in a case–control study of travellers to Thailand after adjusting for age, gender and trip duration, with higher pathogen detection in stool samples with faecal leukocytes or red blood cells.[20] Stratification by Cq values did not improve the association with TD, although this was limited by the sample size of strata.[3] Taken together, these findings suggest that the clinical spectrum of diarrhoeal disease is heterogeneous, and qualitative detection of pathogens in clinically significant TD (i.e. AWD impacting daily activities or invasive TD) is a reliable indicator of TD attribution. An exception to this is EAEC, the most common pathogen detected in asymptomatic controls and loose stool cases and occurred as a co-pathogen in 75% of EAEC-positive TD cases, suggesting that EAEC is less likely to be a primary pathogen. The lower detection rates in our cohort, and variability in the study designs of other travel cohorts makes it difficult to assess the generalizability of our findings. For example, unadjusted OR estimates from other case–control studies have varied based on the region of travel, sampling approach and inclusion of travellers with any diarrhoea (i.e. not meeting TD criteria) as cases or controls.[2,23–25] It is possible that asymptomatic travellers and loose stool cases in our cohort who had higher Cq values (i.e. lower pathogen load) and were more prone to false-negative TAC results from genomic degradation. Although most TD cases and controls were matched by storage duration, 18 pairs were matched without regard to storage duration since we could not find suitable controls. Additional large cohort studies that optimize compliance and reduce the storage interval between FTA card collection and testing are needed to confirm our findings before they can be applied to clinical care.

An important limitation was the sub-optimal compliance with illness diaries (66.7%) and FTA Cards (53.1% in TD cases) despite providing written and verbal instructions and a pre-paid envelope for mailing specimens. A travel duration of <20 days was associated with higher compliance (75%), suggesting that targeting TD surveillance efforts within the first 3–4 weeks of travel is optimal since the median interval between departure and TD onset was 12 days (IQR: 5–37 days). The increasing use of smartphones during travel can facilitate the use of Research Electronic Data Capture for managing digital surveys, which offers advantages, such as minimizing completion time by auto filling certain data (e.g. dates), dynamically hiding or showing fields based on participant input, sending automated text messages at days/times that are convenient for subjects inquiring about symptoms and reminding them to collect FTA Cards and providing subject-specific data visualizations. Implementing such measures to maintain compliance >80% is important since attrition can lead to bias and increased variance that can reduce internal validity.

Conclusion

In conclusion, unsupervised FTA Card and symptom data collection by travellers is a useful adjunct to traditional stool collection methods in resource-limited settings. We observed lower detection rates compared to studies using TAC or stool or FTA Cards prepared by laboratory personnel possibly due to genomic degradation with prolonged storage of FTA Cards or inadequate stool collection. We plan to reduce the storage interval and implement measures to improve compliance in future studies.

Supplementary data

Supplementary data are available at *JTM* online.

Authors' contributions

M.D.T., T.L., I.M., J.A.F. and D.R.T. took care of the concept and design. M.D.T., T.L., I.M., J.A.F., D.R.T., K.T., H.-C.K., D.H.T., A.N.K., H.C.Y. and C.C.G. took care of acquisition, analysis or interpretation of data. M.D.T., T.L. and K.T. were in charge of drafting of the manuscript. All authors took care of the critical revision of the manuscript for important intellectual content. Statistical analysis was done by K.T., I.M. and H.-C.K.

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Research disclaimer

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