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ORIGINAL RESEARCH

Bacterial enteropathogens associated with diarrhea in a rural population of Haiti

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Purpose: Diarrheal disease is one of the leading causes of morbidity in developing countries. To further understand the epidemiology of diarrheal disease among a rural population surrounding Robillard, Haiti, fecal swabs from patients with diarrhea were screened for the presence of enteropathogenic bacteria.

Patients and methods: Fecal swabs were collected from 34 patients with signs and symptoms of diarrhea and stored in BBL™ Cary-Blair transport medium (Becton, Dickinson and Company, Sparks, MD) until transit to the USA. Swab material was inoculated on to different enrichment and selective agars for incubation. Fermenting and nonfermenting bacteria that grew on the enteric selection media were identified by the BBL™ Crystal™ Enteric/Nonferementing Identification system (Becton, Dickinson and Company). Organisms identified as *Escherichia coli* were further screened for the presence of virulence factors by polymerase chain reaction (PCR).

Results: Of 34 patients, no *Campylobacter*, *Shigella*, *Salmonella*, or *Vibrio* spp. were isolated from swabs transported to the USA for culture. Of 73 *E. coli* isolates cultured from the swabs, one enteropathogenic strain of *E. coli* was identified by multiplex PCR. *Escherichia fergusonii* and *Cronobacter sakazakii*, both potential gastrointestinal pathogens, were also isolated from patient stools.

Conclusion: This study was undertaken to determine if bacterial enteropathogens could be detected in the stools of patients suffering from diarrhea or dysentery and, in the absence of sufficient facilities, rectal swabs could be transported to the USA for culture. Although several genera of overt enteropathogens were not detected, one enteropathogenic *E. coli* and other pathogenic enterobacteriaceae were successfully cultured and identified.

Keywords: *Escherichia*, *Cronobacter,* diarrheagenic, stool

Introduction

Diarrheal illness continues to be a major source of morbidity and mortality in the developing world. Accurate figures can be difficult to obtain as many developing nations do not have comprehensive programs for vital statistics.[1] In Haiti, a particular concern is that, after acute lower respiratory infections, diarrheal diseases are the second leading cause of death in children aged under 5 years.[2] The most recent studies indicate that Haiti, in fact, outranks all other countries in the Western hemisphere in under-five mortality, with 102.6 deaths per 1000 births.[3] It is estimated that diarrhea accounted for 17% of under-five mortality worldwide in 2004 and 22% in the developing world in 2000.[4,5] These statistics closely mirror the most recent investigations into under-five mortality in the rural Albert Schweitzer Hospital service area of Haiti, where 20.6% of deaths were recorded as being due to enteric disease.[6] Moreover, diarrhea is one

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of the most common symptoms of acquired immunodeficiency syndrome in developing countries including Haiti, where >95% of patients with acquired immunodeficiency syndrome initially exhibit diarrhea.[7,8]

Before the cholera outbreak in October 2010, there were very few studies investigating the etiology of diarrheal illness in the rural population, where poor sanitation and lack of potable water are prevalent.[9] Many cases of diarrhea with fever are identified as typhoid fever based on symptoms without verification of the diagnosis.[10] Previous studies, such as that by Klipstein et al in 1976, are now outdated due to the multitude of changes that have occurred in Haiti's government, public health programs, and population in the many years since the studies were performed.[11] In an attempt to collect more up-to-date data, the authors of the present study sought to use conventional laboratory media and current molecular techniques, such as polymerase chain reaction (PCR) and genetic sequencing, to investigate potential bacterial causes of diarrhea in a representative sample of a rural population in Haiti.

Materials and methods

Patients and study site

The 34 patients in the study included men, women, children, and infants from the village of Robillard, Haiti, and its environs, located approximately 20 km east of Cap-Haïtien in Nord, Haiti. Informed consent was obtained from all participants and from parents or legal guardians of minors. The study protocols and all associated documentation were prepared and approved by the Institutional Review Board of Middle Tennessee State University prior to the initiation of the investigation. The study was also approved by the pastor of Notre Dame de la Merci parish in Robillard who helped to establish the Centre de Santé Rose-Merci medical clinic in Robillard and served as its director. All patients in the study volunteered to participate upon being seen at the clinic, after presenting with symptoms of diarrhea or dysentery. "Diarrhea" was defined as having multiple loose stools and "dysentery" as having a loose stool containing blood.

Stool sample collection and culturing

Stool samples were collected from participants with a rectal swab that was stored in Cary-Blair transport medium at room temperature. The samples were shipped from Haiti to Middle Tennessee State University under a permit issued by the Centers for Disease Control and Prevention. Total storage and transport time was approximately 3 weeks. Upon arrival, the samples were used to inoculate alkaline peptone water for possible *Vibrio* spp. and brain–heart infusion broth (Becton, Dickinson and Company, Sparks, MD) to enrich facultative and obligate aerobes. Brain–heart infusion broth cultures were grown overnight at 37°C and used to streak tryptic soy agar with 5% sheep blood, MacConkey agar, sorbitol MacConkey agar plates, and Hektoen enteric agar (all from Becton, Dickinson and Company). Alkaline peptone water cultures were streaked onto thiosulfate citrate bile salts sucrose agar (Becton, Dickinson and Company). All of these inoculated media were incubated at 37°C until either visible growth was observed or a maximum of 96 hours had passed. Samples were also used to streak *Campylobacter* agar with five antimicrobics and 10% sheep blood (Becton, Dickinson and Company) that was incubated in a microaerophilic environment at 42°C for the potential identification of *Campylobacter* spp.

Identification of bacteria

After growth on culture media, distinct colonies were selected and identified using the BBL™ Crystal™ Identification system (Becton, Dickinson and Company) for enteric/nonfermenting bacteria. To identify potential diarrheagenic *Escherichia coli* pathogens, a PCR method, as described by Vidal et al, was employed on all *E. coli* colonies.[12] Ten primer pairs (Table 1) were used to amplify virulence genes found in enterotoxigenic *E. coli*, enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli*, enteroaggregative *E. coli*, diffusely adherent *E. coli*, and enteroinvasive *E. coli*.

Results

Patients from this study were examined by physicians of a US medical mission team at the Centre de Santé Rose-Merci medical clinic. Patients were from Robillard and the surrounding area encompassing the Notre Dame de Merci parish. Approximately 1470 patients were seen during the 5-day clinic with 34 patients with diarrhea participating in the study. All patients attending the clinic received the antiprotozoan drug, albendazole, and those with diarrhea received either ciprofloxacin or azithromycin.

In total, 240 potentially distinct bacterial isolates were analyzed via the BBL Crystal Identification system and PCR. Fifteen different bacterial species were able to be identified from the culture media, as shown in Table 2. Of all the isolates analyzed, only a single, overt diarrheagenic pathogen was identified. This isolate was identified as *E. coli* by the BBL Crystal Identification system and genes for the bundle forming pilus (*bfp*) and intimin (*eae*) were amplified

Table 1 PCR primers used to amplify bacterial virulence genes in *Escherichia coli*

Gene	Associated diarrheagenic *Escherichia coli*	Primer sequences (5′–3′)[a]	Size of PCR product (bp)
stx1	EHEC	CAG TTA ATG TGG TGG CGA AGG CAC CAG ACA ATG TAA CCG CTG	348
stx2	EHEC	ATC CTA TTC CCG GGA GTT TAC G GCG TCA TCG TAT ACA CAG GAG C	584
eae	EHEC, EPEC	TCA ATG CAG TTC CGT TAT CAG TT GTA AAG TCC GTT ACC CCA ACC TG	482
bfp	EPEC	GGA AGT CAA ATT CAT GGG GGT AT GGA ATC AGA CGC AGA CTG GTA GT	300
lt	ETEC	GCA CAC GGA GCT CCT CAG TC TCC TTC ATC CTT TCA ATG GCT TT	218
stII	ETEC	AAA GGA GAG CTT CGT CAC ATT TT AAT GTC CGT CTT GCG TTA GGA C	129
virF	EIEC	AGC TCA GGC AAT GAA ACT TTG AC TGG GCT TGA TAT TCC GAT AAG TC	618
ipaH	EIEC	CTC GGC ACG TTT TAA TAG TCT GG GTG GAG AGC TGA AGT TTC TCT GC	933
daaE	DAEC	GAA CGT TGG TTA ATG TGG GGT AA TAT TCA CCG GTC GGT TAT CAG T	542
aafII	EAEC	CAC AGG CAA CTG AAA TAA GTC TGG ATT CCC ATG ATG TCA AGC ACT TC	378

Notes: [a]Upper sequence indicates the forward primer and the lower sequence indicates reverse primer. All primer sequences are from Vidal et al.[12]
Abbreviations: PCR, polymerase chain reaction; ETEC, enterotoxigenic *E. coli*; EPEC, enteropathogenic *E. coli*; EHEC, enterohemorrhagic *E. coli*; EAEC, enteroaggregative *E. coli*; DAEC, diffusely adherent *E. coli*; EIEC, enteroinvasive *E. coli*.

with PCR (Figure 1). The presence of these genes allowed for a presumptive identification of EPEC, which was later confirmed by DNA sequencing of the PCR products. This pathogenic strain was isolated from a sample taken from a 7-month-old female. Also of note was the identification of *Escherichia fergusonii* in three patient samples. *E. fergusonii* has been associated with diarrheal illness in goats and clinical cases that resemble salmonellosis.[13,14] It has also been reported that *E. fergusonii* is an emerging pathogen in parts of India, although the cases there were primarily associated with wound and urinary tract infections.[15] Additionally, *Cronobacter sakazakii* was isolated from the stool of a 4-month-old male with diarrhea. *C. sakazakii* has been associated with necrotizing enterocolitis in infants.[16] All of the isolates were members of the Enterobacteriaceae, and most

Table 2 Bacterial species isolated from 34 patients with diarrhea and their rate of occurrence

Bacterial isolate	Positive patients (%)
Escherichia coli	34 (100)
Klebsiella oxytoca	19 (55.9)
Klebsiella pneumoniae	13 (38.2)
Citrobacter freundii	12 (35.3)
Enterobacter cloacae	8 (23.5)
Morganella morganii	6 (17.6)
Citrobacter koseri	4 (11.8)
Kluyvera ascorbata	4 (11.8)
Escherichia fergusonii	3 (8.8)
Proteus mirabilis	3 (8.8)
Proteus vulgaris	3 (8.8)
Providencia alcalifaciens	2 (5.9)
Cronobacter sakazakii	1 (2.9)
Enterobacter aerogenes	1 (2.9)
Kluyvera cryocrescens	1 (2.9)

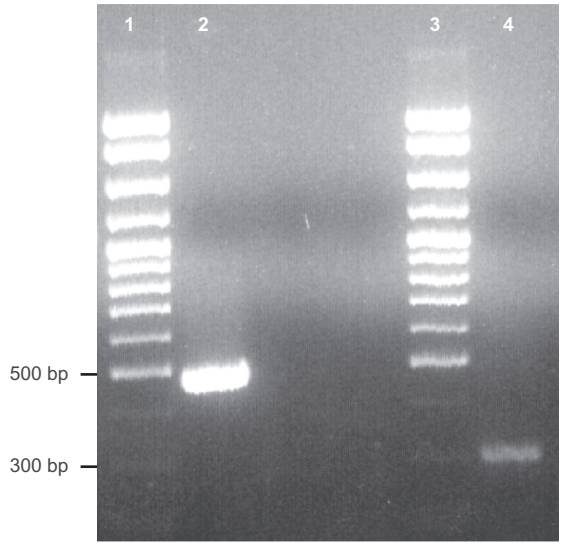

Figure 1 One percent Tris-acetate-ethylenediaminetetraacetic acid agarose gel with ethidium bromide staining of polymerase chain reaction (PCR) products from enteropathogenic *Escherichia coli* isolate. Lane 1, molecular weight markers; lane 2, 482 bp PCR product from an *E. coli* isolate corresponding to the *eae* gene; lane 3, molecular weight markers; lane 4, 300 bp PCR product from the same *E. coli* isolate corresponding to the *bfp* gene. Both *eae* and *bfp* genes are present in enteropathogenic *Escherichia coli* strains.

of these likely represent normal intestinal microbiota. No *Campylobacter*, *Shigella*, *Salmonella*, or *Vibrio* spp. were isolated from any of the swabs. Although cases of typhoid fever have been reported in Robillard, no other bacterial causes of bacterial diarrhea had been diagnosed in the village in recent history.[17] Nonetheless, those reporting diarrhea did receive antibiotic therapy.

Discussion

While this study was unsuccessful in identifying a significant number of bacterial enteropathogens in those experiencing symptomatic diarrhea or dysentery in Robillard, Haiti, the authors feel that they have demonstrated that, in principle, it is feasible to collect clinical samples in remote regions of developing countries such as Haiti and analyze them in laboratories elsewhere.

Campylobacter, *Shigella*, *Salmonella*, *Yersinia*, and *Vibrio* spp. are all epidemiologically important bacterial causes of diarrhea. However, because none of these bacteria were isolated from the transported swabs, it is probable that some bacterial species were lost en route. *Aeromonas* and *Plesiomonas* spp. can also be responsible for diarrhea and may persist in drinking water sources; however, none of these bacteria were isolated and identified. The presence of pathogenic anaerobes such as *Clostridum* spp. that have been reported as bacterial agents of diarrhea in developing countries were also not assessed.[8] Other limitations to the recovery of these organisms may have been the collection of only a single rectal swab compared with collecting multiple samples or stool specimens. However, because these samples were to be transported to the USA, swabs in Cary-Blair transport medium were considered the best option.[18] The possibility that some of the *E. coli* bacterial isolates could have been pathogenic but lost genetic elements containing virulence genes during transit was also considered, however, the single EPEC isolate identified retained its large EPEC-adherence factor plasmid containing the *bfp* gene. This suggests that pathogenic *E. coli* can potentially be a cause of disease in rural villages. In a 1995 report on the microbiological laboratory results of the US Army's 86th Combat Support Hospital, only 16% of stool cultures from patients who primarily exhibited watery diarrhea characteristic of traveler's diarrhea tested positive for an enteric pathogen with only *Shigella* sp. and *Plesiomonas shigelloides* being detected.[19] None of the patients were tested for pathogenic *E. coli*. As the current cholera epidemic subsides, knowledge that bacteria such as EPEC and other lactose-fermenting Enterobacteriaceae (the signs and symptoms of which can be mistaken for cholera, typhoid fever, or nonbacterial diseases, such as rotavirus or cryptosporidiosis) are also potential pathogens for the population of Haiti may lead to more appropriate therapies, especially for children.

At the time of this study, approximately 10% of the population attending the clinic received its water from a single spring-fed spigot (Figure 2). The population surrounding Robillard obtained water from proximate water sources consisting of streams and pools. A previous team (unpublished data) had shown that both the water from this spigot and surrounding streams were contaminated with fecal indicator bacteria. It is likely that these water sources are the reservoirs for diarrheagenic pathogens that are also shared by populations of domestic animals. In April 2011, the population of Robillard and its environs in Nord, Haiti, began experiencing an outbreak of cholera. A better understanding of the types of bacteria that potentially cause diarrhea and the sources of these bacteria can assist medical personnel with limited diagnostic facilities to more effectively treat the population.

This study has also provided valuable insight into the difficulties one must overcome to perform comprehensive clinical examinations in a rural setting where there is only intermittent electricity via generators and little or no laboratory equipment.

Future studies should include a larger sample size from multiple sites that more accurately represents the rural population of Haiti, as well as samples from individuals not experiencing diarrhea. Additionally, given the significant contamination of rural water sources, rapid tests should be used onsite to facilitate the identification of a wider range of potential causes for diarrheal disease in drinking water sources.

Figure 2 The single spigot in the village of Robillard, Nord, Haiti, from which villagers draw water for drinking and cooking.

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Disclosure

The authors report no conflicts of interest in this work.

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