Original Article

Antibiotic resistant *Shigella* is a major cause of diarrhoea in the Highlands of Papua New Guinea

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Abstract

Introduction: Diarrhoea remains a major cause of illness in Papua New Guinea (PNG); however, little is known about its aetiology. As a result of the cholera outbreak that spread throughout PNG in 2009-2011, we conducted diarrhoeal surveillance in Eastern Highlands Province.

Methodology: Following informed consent and a brief questionnaire, participants provided a stool sample or duplicate rectal swabs. Samples were tested for common bacterial pathogens *Salmonella* spp., *Shigella* spp., *Vibrio* spp., *Campylobacter* spp. and *Yersinia enterocolitica* using established culture methods. Enteric parasites were detected using microscopy.

Results: A total of 216 participants were enrolled; where age was recorded, 42% were under 5 years of age, 6.7% were 5 to 17 years of age and 51.3% ≥18 years of age. One or more pathogens were detected in 68 (31.5%) participants, with *Shigella* (primarily *S. flexneri*) being the most commonly isolated (47 of 216 participants). Enteric parasites were detected in 23 of the 216 participants, occurring as a co-infection with another pathogen in 12 of 23 cases. No *Vibrio cholerae* was detected. *Shigella* isolates were commonly resistant to ampicillin, tetracycline, co-trimoxazole and chloramphenicol.

Conclusions: Shigellae, specifically *S. flexneri*, are important pathogens in the highlands of PNG. While most studies in low-income settings focus on childhood aetiology, we have demonstrated the importance of *Shigella* in both children and adults. Enteric parasites remain present and presumably contribute to the burden of gastrointestinal illness. While improvements in sanitation and hygiene would help lower the burden of all aetiologies of infectious diarrhoea, additional control strategies targeting *Shigella* may also be warranted.

Key words: *Shigella*; diarrhoea; low-income; enteric disease.

J Infect Dev Ctries 2014; 8(11):1391-1397. doi:10.3855/jidc.4396

(Received 07 November 2013 – Accepted 22 January 2014)

Introduction

The global burden of enteric diseases remains unacceptably high, despite improvements in recent years. Diarrhoea remains the second leading cause of mortality in children under the age of 5 years with the burden greatest in low-income settings [1], where poor sanitation, hygiene and food safety facilitate the spread of enteric pathogens. A recent multi-centre case-control study found rotavirus, *Cryptosporidium*, enterotoxigenic *Escherichia coli* and *Shigella* spp. to have a significant association with moderate-severe diarrhoea in children at all study sites [2]. The findings may be indicative of the aetiology of enteric illness in many low-income settings; but regional variations in diarrhoeal aetiology are known to occur, as recognized in the multicentre study [2]. Such studies are valuable and set the framework for global research and intervention priorities, but do not negate the need for country specific data.

In Papua New Guinea (PNG), government and non-government organisations quote figures that suggest diarrhoea and other enteric illness (primarily typhoid fever) remain amongst the most important causes of morbidity and mortality [3,4]. However, the current data are weak as there have been very few studies conducted in recent times investigating the aetiology of diarrhoea in PNG. The most recent comprehensive data in PNG comes from a study conducted in children in 1985-1990 [5]. More recently Horwood and colleagues conducted rotavirus surveillance in children hospitalized with acute watery diarrhoea in the highlands of PNG. Rotavirus was detected in 31.2% of study participants, demonstrating its importance in childhood gastroenteritis. However, other causes of diarrhoea were not sought [6].

Globally most studies focus on childhood diarrhoea, as the burden of disease and the potential for severe negative outcomes is greatest in this age group (< 5 years). However, diarrhoeal illnesses also affect adults, and the all-age global burden of diarrhoea is considerable [7,8]. Recent outbreaks of shigellosis [9] and cholera [10,11] in PNG have demonstrated the impact diarrhoeal diseases can have on the adult population. The recent cholera epidemic was the first outbreak of this disease in PNG and the response at the national level was hampered by a lack of diagnostic capacity [12]. As part of the response to the cholera outbreak, we conducted surveillance of diarrhoea in children and adults in the Eastern Highlands of PNG at a time when sporadic cases of cholera had been reported in highland provinces.

Methodology

Informed Consent, Recruitment and Sample Collection

Adults and children presenting to Lopi Urban Clinic and Goroka General Hospital out-patients with self-reported diarrhoea were invited to participate in the study. The study was approved by the PNG Institute of Medical Research Institutional Review Board (IRB 0926) and the PNG Medical Research Advisory Committee (MRAC 10.09). Following informed consent, participants were interviewed and a questionnaire completed by research staff. The questionnaire provided demographic information for each participant. When possible a stool sample was provided: if the patient was unable to provide a stool sample duplicate rectal swabs were collected. Rectal swabs were placed into Cary-Blair transport medium and stool samples were collected in stool specimen jars. All specimens were held at room temperature until they could be transported to the laboratory (within 4 hours of sample collection).

Detection of pathogens

Upon arrival at the laboratory specimens were processed immediately. Culture was conducted for the bacterial pathogens, specifically targeting *Salmonella* spp., *Shigella* spp., *Vibrio* spp., *Yersinia enterocolitica* and *Campylobacter* spp. MacConkey agar, Xylose Lactose Desoxycholate (XLD) agar, thiosulphate citrate bile salts sucrose (TCBS) agar, *Yersinia* selective agar (with cefsulodin 15mg/l, Irgasan 4 mg/l and novobiocin 2.5 mg/l) and *Campylobacter* blood-free selective agar with CCDA selective supplement (cefoperazone 32 mg/l and amphotericin B 10 mg/l) (Thermo Fisher Scientific, Scoresby, Australia) were used for the selective isolation of the targeted bacterial pathogens. Following inoculation of plates, a small portion (~1 g) of stool, or the swab that had been used to inoculate the agar plates, was placed into mannitol selenite cysteine broth and incubated at 37°C for 18-24 hours to enrich for *Salmonella* spp. The enrichment broth was then plated onto XLD agar. All plates were incubated at 37°C for 24 hours initially, and a further 24 hours if required. *Campylobacter* plates were incubated under microaerophilic conditions (CampyGen, Thermo Scientific, Scoresby, Australia); all other plates were incubated aerobically.

Biochemical tests were conducted on suspect colonies using standard procedures [13] and, where necessary, confirmed using the Crystal biochemical profiling system (BD Pty Ltd, Sydney, Australia). Suspected isolates of *Shigella* and *Salmonella* were confirmed by real-time PCR [14-16] and polyvalent antisera when available (Remel, Thermo Scientific, Scoresby, Australia).

An iodine-stained wet prep was viewed under a microscope to detect parasites according to standard techniques [17]. When available a portion of a stool sample was used; or alternatively the second of the two rectal swabs provided. *Entamoeba histolytica* was differentiated from *Entamoeba coli* on the basis of cyst size using a calibrated graduated eye piece.

Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was conducted using the Kirby-Bauer disk diffusion method on all isolated bacterial pathogens. Isolates were tested for susceptibility/resistance to ampicillin, chloramphenicol, ciprofloxacin, ceftriaxone, nalidixic acid, co-trimoxazole and tetracycline, following appropriate guidelines [18]. Isolates were preserved in skim milk glucose glycerol broth and stored at -80°C. At a later date viable isolates were resuscitated and the minimal inhibitory concentration (MIC) was determined using E-test strips (BioMérieux, Baulkham Hills, Australia).

Data Entry and Analysis

Data were entered into Microsoft Excel (Microsoft Corporation, Redmond, USA) and analysis conducted using Excel and SPSS Statistics 20 (IBM Corporation, Armonk, USA).

Results

A total of 216 samples were collected from October 2010 to August 2011 and included in analysis. Age was recorded for 195 of the 216 participants, with approximately half being adults and half under the age

of 18 years. Males more commonly presented than females. The age and sex distributions are shown in Table 1. Of the 195 participants whose age was recorded, one or more pathogens were detected in 66 participants: 43 of 100 (43%) adult participants; 4 of 13 (30.8%) in the 5 to 17 years of age group; and 19 of 82 (23.2%) in children under 5 years of age. The rate of pathogen isolation differed significantly between adults and all children (≤ 17) (χ^2 = 7.682, degrees freedom 1, p = 0.005) and adults and children less than 5 years old (χ^2 = 7.887, degrees freedom 1, p = 0.005).

Pathogens were isolated from 68 of 216 (31.5%) study participants (66 pathogens from people with age recorded and an additional 2 pathogens from participants without age recorded): 55 participants had a single pathogen isolated and 13 had two or more pathogens isolated. *Shigella* spp. were the most common pathogen isolated, being present in 47 (21.8%) samples. Of the 47 *Shigella* isolates, 32 were speciated by serology: the majority (30/32) were *S. flexneri* and the remaining two were serologically confirmed as isolates of *S. dysenteriae*. Five samples (2.3%) were culture positive for *Salmonella enterica* serovar Typhi. No other bacterial pathogens were detected. *Entamoeba histolytica* (n = 15; 6.9 %), *Blastocystis hominis* (n = 10; 4.6%) and *Giardia lamblia* (n = 7; 3.2%) were the most commonly detected parasites. Table 2 provides a summary of the detection of pathogens.

Patients self-reported on the characteristics of their stools in the days leading up to presentation. Of the 216 patients in the study, 61 reported blood in their stools and 81 reported having mucus present. Patients with confirmed shigellosis or amoebiasis were more likely to report having had blood in their stools than all other patients, although the difference was not significant to the P < 0.05 level (P = 0.098). No relationships were observed for the self-reported presence of mucus in stools. Patients who reported having diarrhoea for at least 1 week were more likely

Table 1. Age and sex distribution of study participants that had both data recorded (195 of 216 participants).

	< 5yrs	**5 - 17 years**	**≥ 18years**
N (%)	82 (42%)	13 (6.7%)	100 (51.3%)
Sex (M:F)	44:35*	7:6	66:34

*Sex was not recorded for 3 children <5 years old.

Table 2. Detection of enteric pathogens, with percentage prevalence in parenthesis, according to age group in the highlands of Papua New Guinea.

Pathogen	**< 5 years n = 82**	**5 – 17 years n = 13**	**≥18 years n = 100**	**Total n = 216***
Shigella flexneri	6 (7.3)	2 (15.4)	22 (22)	30 (13.9)
Shigella dysenteriae	1 (1.2)	0 (0)	1 (1)	2 (0.9)
Shigella (not typed)	9 (11.1)	0 (0)	5 (5)	15† (6.9)
Salmonella Typhi	1 (1.2)	1 (7.7)	2 (2)	5† (2.3)
Entamoeba histolytica	1 (1.2)	1 (7.7)	13 (13)	15 (6.9)
Blastocystis hominis	1 (1.2)	0 (0)	9 (9)	10 (4.6)
Giardia lamblia	2 (2.4)	1 (7.7)	4 (4)	7 (3.2)
Ascaris lumbricoides	0 (0)	0 (0)	1 (1)	1 (0.5)
Hookworm	0 (0)	0 (0)	1 (1)	1 (0.5)
Multiple pathogens	1 (1.2)	1 (7.7)	11 (11)	13 (6.0)

*Includes participants whose age was not recorded; †One additional *Shigella* sp. isolate and one additional *S.* Typhi isolate from study participants who did not have age recorded.

Table 3. Correlations between the presence of blood or mucus in stool and detection of recognised dysenteric pathogens, and between duration of illness at time of presentation and the detection of any pathogen.

	Shigella* and/or *E. histolytica	**Any pathogen**	**analysis**
Blood (n = 61)	21	NA	p = 0.098
No blood (n = 154)	36	NA	(χ^2 = 2.738, df = 1)
Mucus (n = 81)	23	NA	p = 0.626
No mucus (n = 134)	34	NA	(χ^2 = 0.237, df = 1)
< 1 week (n = 111)	NA	27	p = 0.02
> 1 week (n = 105)	NA	41	(χ^2 = 4.761, df = 1)

For presence of blood or mucus in stools, one participant did not respond, thus total of 215

to have a pathogen detected than those with diarrhoea for less than a week (P = 0.02). Table 3 provides details of statistical analyses.

Antibiotic Susceptibility Testing

Both *Shigella* and *S.* Typhi were commonly resistant to ampicillin, tetracycline, co-trimoxazole and chloramphenicol. No isolates were resistant to ciprofloxacin or ceftriaxone, and only one *S.* Typhi isolate was resistant to nalidixic acid. Multiple resistance was common, with over half (26 of 47) of all *Shigella* isolates resistant to four antibiotics (ampicillin, tetracycline, co-trimoxazole and chloramphenicol). Antibiotic resistance data are provided in Table 4.

Minimum inhibitory concentrations (MICs) were conducted on *Shigella* isolates, with MIC results corroborating disk diffusion results. High levels of resistance were commonly observed: for ampicillin, 16 of 29 isolates tested had an MIC >256 μg/ml; for tetracycline, 10 of 25 isolates tested had an MIC >256 μg/ml; for cotrimoxazole, 22 of 22 isolates tested had an MIC >32 μg/ml; and for chloramphenicol, 6 of 27 isolates tested had an MIC >256 μg/ml.

Discussion

Our study highlights the importance of *Shigella* as an enteric pathogen in both children and adults in the highlands of PNG, with *S. flexneri* being the predominant species. This is the first study in the past 25 years to seek multiple aetiologies in diarrhoeal cases in PNG, and the first such study in adults (to our knowledge). The importance of *Shigella* as an enteric pathogen in PNG reflects findings in other low-income settings. A recent study in both children and adults with diarrhoea in Ethiopia isolated *Shigella* from 15.6% of participants, with enteric parasite co-infections occurring in 18.3% of shigellosis cases [19]. In our study, *Shigella* and intestinal parasite co-infections were observed in 10.6% of shigellosis cases.

The isolation rate of *Shigella* in this study was higher than that of Howard and colleagues [5] in children in the same setting between 1985 and 1990 (13%), although direct comparisons are difficult due to differences in study design and target age groups. However, our isolation rate in children less than 5 years of age (19.5%) was higher than in symptomatic children in the study conducted by Howard *et al*. [5]. On the basis of these data, it would seem that the burden of *Shigella* has not diminished in PNG in the past 25 years.

Approximately 20 years ago typhoid fever was highly endemic in the highlands of PNG, with an incidence rate among the highest in the world at that time [20]. A recent evaluation of typhoid fever diagnostic tests detected *S.* Typhi in ~8% of febrile patients [21]. It is well recognised that *S.* Typhi can be carried asymptomatically; thus it is not possible to ascertain whether *S.* Typhi was the cause of diarrhoea in our culture positive study participants. However, in this setting diarrhoea is commonly associated with typhoid fever, with a high proportion of confirmed typhoid fever positive patients in the recent diagnostic evaluation [21] self-reporting diarrhoea as a symptom (unpublished data). Regardless of whether *S.* Typhi contributed to diarrhoea, our data provide further evidence that *S.* Typhi continues to circulate in this population and contribute to the overall burden of enteric diseases.

We did not detect any non-typhoid *Salmonella*

Table 4. Proportion of *Shigella* spp and *S.* Typhi isolates resistant to antibiotics, and proportion of multiple-resistant strains.

	***Shigella* spp. n = 47 (%)**			***Salmonella* Typhi n = 5 (%)**		
	R	**I**	**S**	**R**	**I**	**S**
Ampicillin	43 (91.5)	0 (0)	4 (8.5)	4 (80)	0 (0)	1 (20)
Tetracycline	36 (76.6)	0 (0)	11 (23.4)	3 (60)	0 (0)	2 (40)
Co-trimoxazole	33 (70.2)	1 (2.1)	13 (27.7)	3 (60)	0 (0)	2 (40)
Chloramphenicol	26 (55.3)	11 (23.4)	10 (21.3)	2 (40)	0 (0)	3 (60)
Naladixic acid	0 (0)	0 (0)	47 (100)	1 (20)	0 (0)	4 (80)
		Multiple resistance			Multiple resistance	
5 antibiotics		0 (0%)			1 (20%)	
4 antibiotics		26 (55.3%)			1 (20%)	
3 antibiotics		10 (21.3%)			1 (20%)	
2 antibiotics		7 (14.9%)			0 (0%)	
1 antibiotic		2 (4.3%)			1 (20%)	
Fully susceptible		2 (4.3%)			1 (20%)	

R - resistant; I - intermediate resistance; S - susceptible

(NTS) in this study. *Salmonella* are well recognised gastrointestinal pathogens, and NTS has become one of the most important pathogens in AIDS patients in sub-Saharan Africa. NTS has been detected in foods in lowland PNG [22], but it does not seem to play such a major role in enteric or febrile illness in highland PNG. The study conducted by Howard and colleagues detected *Salmonella* spp. in 4% of children with diarrhoea, most commonly in 3 – 11 year olds (9% isolation rate in cases). We had only 15 participants in that age range in our current study. A more recent study looking at the cause of mortality in children in this setting isolated *Salmonella* from 2 of 354 participants [23]; however stool culture was not conducted in that study and those isolates were from blood or cerebral spinal fluid culture where the isolation rate of NTS would be expected to be lower. Nonetheless, our failure to isolate NTS is not appreciably different to that of previous findings in this setting. Moreover, we have not detected NTS in febrile patients in the general population [21] or in the HIV-positive population in this setting (unpublished data).

Antibiotic resistance was common in *Shigella* spp. isolated in the current study. Almost all isolates (91.5%) were resistant to ampicillin, with resistance to tetracycline, co-trimoxazole and chloramphenicol also common. Every isolate that was resistant to chloramphenicol (26 of 47) was also resistant to the three other antibiotics above. These findings are consistent with recent observations in PNG and other low-income settings [24-27]. Multiple antibiotic resistance was also observed in *S*. Typhi. Moreover, two of the five isolates were resistant to chloramphenicol, the first line antibiotic for typhoid fever in PNG [28,29]. One *S*. Typhi isolate exhibited resistance to nalidixic acid. No *Shigella* isolates were resistant to ciprofloxacin or nalidixic acid, which is encouraging given the current national guidelines recommend ciprofloxacin as the first-line antibiotic for dysentery in cases were antibiotic therapy is required [28,29]. Globally there is increasing resistance to quinolones in *Shigella* spp. [30], thus ongoing surveillance in PNG is required.

We detected parasites in 10.6% of participants. This detection rate is lower than in healthy pregnant women in the same area of PNG where comparable methods were used [31], and also lower than in a recent comparable study conducted in Ethiopia [19]. The true burden of parasites may not have been detected in this study. Wet preps are of most diagnostic value when conducted on fresh stools, while trophozoites are still motile [32]. Due to limitations in resources and capacity, stool samples could not be preserved (in sodium acetate-acetic acid-formalin solution or similar) immediately following sample collection. Adding to the problem is the cultural reluctance to give stool samples in PNG: in most cases in this study rectal swabs were given rather than stools. Newer technologies, such as molecular based approaches, may be appropriate in low-income settings to determine the true prevalence of intestinal parasite infections.

Pathogens were more commonly detected in adults than in children. This may be, in part, an artefact of the limited target organisms in this study. The burden of rotavirus in this setting has recently been reported [6], and other pathogens not targeted in this study are commonly associated with childhood diarrhoea, *e.g.* enteropathogenic *Escherichia coli*, isolated from 8% of cases by Howard *et al*. [5].

In this study we used molecular detection of the ipaH gene to confirm isolates as *Shigella* sp. This is a digression from the recognised practice of confirmation of *Shigella* through serology [33]. Using molecular methods we are unable to speciate *Shigella* as all four 'species' are genetically very similar. We found this method to be a viable alternative to traditional *Shigella* confirmatory testing in this setting which costs less to conduct per test (for a laboratory with the appropriate equipment). Although the ipaH gene is also present in enteroinvasive *Escherichia coli* (EIEC), this assay was used as a confirmatory test only, not for direct detection in stool samples. As such, we were able to rule out EIEC on the basis of colony morphology and biochemical tests.

We did not detect evidence of ongoing transmission of cholera in the highlands of PNG. We have reported on the epidemiology of cholera in PNG elsewhere; with transmission occurring only in lowland and island regions of PNG [10]. However, this study did highlight the importance of *Shigella* in gastrointestinal illness in all age groups in the highlands of PNG. These findings support historical and recent data that demonstrate *Shigella* to be one of the most important gastrointestinal pathogens. Concerted efforts are required globally to lessen the burden of this pathogen.

Acknowledgements

The authors acknowledge the input of the study nurses, Rebecca Sehuko and Anna Solomon. We also acknowledge the support of management and staff at Goroka General Hospital and Lopi Urban Clinic. We give thanks to staff

within the Infection and Immunity unit and the Environmental and Emerging Diseases unit at the PNG Institute of Medical Research who assisted with lab work. We dedicate this work to the memory of the late Ms Audrey Michael, a major contributor to this and other infectious disease reserch projects in PNG. This study was partially supported by a Partnership in Health Program grant from Esso Highlands Limited, an ExxonMobil subsidiary.

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Conflict of interest: No conflict of interests is declared.