



## Detection of enteric viral and bacterial pathogens associated with paediatric diarrhoea in Goroka, Papua New Guinea



Kevin W. Soli<sup>a</sup>, Tobias Maure<sup>a</sup>, Monalisa P. Kas<sup>a</sup>, Grace Bande<sup>a</sup>, Sauli Bebes<sup>a</sup>, Dagwin Luang-Suarkia<sup>a</sup>, Peter M. Siba<sup>a</sup>, Ayako Morita<sup>b</sup>, Masahiro Umezaki<sup>b</sup>, Andrew R. Greenhill<sup>a,c</sup>, Paul F. Horwood<sup>a,\*</sup>

<sup>a</sup> Papua New Guinea Institute of Medical Research, PO Box 60, Goroka EHP441, Papua New Guinea

<sup>b</sup> Department of Human Ecology, The University of Tokyo, Tokyo, Japan

<sup>c</sup> School of Applied and Biomedical Sciences, Federation University, Churchill, Victoria, Australia

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### SUMMARY

**Objectives:** The aim of this study was to investigate the viral and bacterial causes of acute watery diarrhoea in hospitalized children in Papua New Guinea.

**Methods:** A retrospective analysis was conducted on stool samples collected from 199 children (age <5 years) admitted to the paediatric ward of Goroka General Hospital from August 2009 through November 2010. A large range of viral and bacterial enteric pathogens were targeted using real-time PCR/RT-PCR assays.

**Results:** Young children were much more likely to be admitted with acute gastroenteritis, with 62.8% of patients aged <1 year and 88.4% aged <2 years. An enteric pathogen was detected in 69.8% ( $n = 138$ ) of patients. The most commonly detected pathogens were *Shigella spp* (26.6%), rotavirus (25.6%), adenovirus types 40/41 (11.6%), enterotoxigenic *Escherichia coli* (11.1%), enteropathogenic *E. coli* (8.5%), norovirus G2 (6.0%), and *Campylobacter spp* (4.0%). Norovirus G1, sapovirus, and *Salmonella spp* were also detected, but below our statistical limit of detection. *Vibrio cholerae* and astrovirus were not detected in any patients. Mixed infections were detected in 22.1% of patients, with *Shigella* and rotavirus most commonly detected in co-infections with other pathogens.

**Conclusions:** This study demonstrates that *Shigella* and rotavirus are the major pathogens associated with acute paediatric gastroenteritis in this setting.

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## 1. Introduction

Diarrhoeal illnesses are a major cause of morbidity and mortality in children worldwide. An estimated 1.8 million children die each year from largely preventable enteric illnesses, with the large majority of these mortalities occurring in developing countries.<sup>1</sup> Rotavirus is the most important cause of acute gastroenteritis in children worldwide, with recent estimates suggesting that more than 1.4 million hospital admissions and 500 000 deaths can be attributed to rotavirus infections annually.<sup>2</sup> However, many viral, bacterial, and protozoan pathogens are associated with diarrhoeal illness in children, and this broad range

of pathogens involved in childhood diarrhoea contributes to challenges in overcoming the high burden of disease.

In Papua New Guinea, enteric illnesses are one of the major causes of outpatient visits, hospitalizations, and mortalities, particularly in young children.<sup>3</sup> However, poor diagnostic capacity has greatly limited knowledge pertaining to the aetiology and epidemiology of enteric illnesses in this setting.<sup>4</sup> Past studies have established rotavirus as an important cause of acute gastroenteritis in children in this setting,<sup>5,6</sup> and outbreaks of cholera<sup>7</sup> and shigellosis<sup>8</sup> have also affected many communities. However, little effort has been made in recent years to fully comprehend the aetiology of childhood enteric illness in Papua New Guinea by way of detection of multiple aetiologies, with the most recent comprehensive study conducted on samples that were collected approximately 25 years ago.<sup>5</sup> In this study, we investigated the

\* Corresponding author. Tel.: +675 532 2800; fax: +675 532 1998.  
E-mail address: [paul.horwood@hotmail.com](mailto:paul.horwood@hotmail.com) (P.F. Horwood).

aetiology of acute watery diarrhoea in hospitalized children in Goroka, Papua New Guinea.

## 2. Materials and methods

### 2.1. Sample collection

Stool samples were collected from children <5 years of age hospitalized with acute gastroenteritis at Goroka General Hospital, Papua New Guinea from August 2009 through November 2010. Goroka is the provincial capital of the Eastern Highlands Province of Papua New Guinea, and Goroka General Hospital is the major referral hospital for the region and has a catchment population of approximately 450 000 people. Children were considered eligible for inclusion in the study if their parent/guardian reported acute watery diarrhoea (>3 loose bowel movements in the last 24 h) and no blood or mucous was observed in the faeces. Dysentery samples were excluded from analysis because sample collection was designed for acute watery diarrhoea surveillance as part of the Western Pacific Region Rotavirus Surveillance Network (World Health Organization). All samples were collected within 48 h of hospitalization and transported to the laboratory at 4 °C and then aliquoted and stored at –80 °C.

### 2.2. Detection of enteric pathogens

Nucleic acids were extracted from the stool samples using the Qiagen DNA Stool Mini Kit (Qiagen, Germany) in accordance with the manufacturer's instructions. Eluates were stored at –80 °C until required for testing.

Eluates from the stool samples were tested for a range of viral and bacterial enteric pathogens using real-time PCR/RT-PCR assays (Table 1).<sup>27–33</sup> RNA viruses (rotavirus, norovirus G1 and G2, sapovirus, and astrovirus) were detected in individual reactions using the QuantiTect Multiplex Real-Time RT-PCR Mastermix (Qiagen, Germany). DNA viruses (adenovirus types 40/41) and bacteria (*Shigella spp*, *Salmonella spp*, *Vibrio cholerae*, *Campylobacter spp*, enteropathogenic *Escherichia coli* (EPEC) and enterotoxigenic *E. coli* (ETEC)) were detected in individual reactions using the QuantiTect Multiplex Real-Time PCR Mastermix (Qiagen, Germany). All reaction conditions were those given in the Mastermix manufacturer's instructions. Negative and positive controls were included in each run to ensure the validity of results.

### 2.3. Sample size and statistics

We calculated sample size based on historical data from this setting, where the most important pathogens were detected in at least 4% of cases.<sup>5</sup> To enable detection of pathogens with a prevalence of 8% to a precision of ±4% at a 95% confidence level, we required a sample size of 177. Pathogen prevalence was calculated in Microsoft Excel and expressed as a percentage of positive samples in relation to the total number of samples tested.

## 3. Results

Stool samples were collected from 199 children <5 years of age (109 male and 90 female) admitted with acute gastroenteritis to Goroka General Hospital. The age range of the children was 1–60 months, with a median age of 10 months. There was no significant difference in the detection of any enteric pathogens between males and females (Fisher's exact test, data not shown). Analysis of the age distribution of infections between children aged <1 year ( $n = 125$ ) and children aged 1–5 years ( $n = 74$ ) showed no significant difference in the detection rate of pathogens between

the two age groups (Fisher's exact test, data not shown). Young children were much more likely to be admitted to hospital with acute gastroenteritis, with 62.8% of patients aged <1 year and 88.4% aged <2 years.

An enteric pathogen was detected in 69.8% ( $n = 138$ ) of stool samples collected from children hospitalized with acute gastroenteritis. Table 2 shows the distribution of pathogens found in the stool samples. *Shigella spp* (26.6%,  $n = 53$ ), rotavirus (25.6%,  $n = 51$ ), and adenovirus 40/41 (11.6%,  $n = 23$ ) were the enteric pathogens most commonly detected. EPEC strains were detected in 8.5% ( $n = 17$ ) of patients. The majority ( $n = 13$ ) of the EPEC strains detected were classified as atypical, based on the absence of the EPEC adherence factor (EAF) plasmid. ETEC strains were detected in 11.1% ( $n = 22$ ) of the samples, with the ETEC toxin genes ST and LT detected in 7% ( $n = 14$ ) and 6% ( $n = 12$ ) of samples, respectively. Both toxin genes were detected in four samples; however, without isolation of *E. coli* strains we cannot ascertain whether multiple strains were present in individual samples or single strains contained both toxin genes. *Campylobacter spp* (4.0%,  $n = 8$ ) was the only other pathogen detected within the limits of detection for this study; other enteric pathogens such as norovirus G1, norovirus G2, sapovirus, and *Salmonella spp* occurred sporadically (in <10% of patients) but below our statistical limit of detection. Astrovirus and *V. cholerae* were not detected in any patients. Mixed infections were detected in 22.1% ( $n = 44$ ) of patients, with *Shigella* (13.1%,  $n = 26$ ) and rotavirus (11.1%,  $n = 22$ ) the pathogens most commonly detected in mixed infections. Other co-infections were also detected at a high frequency (Tables 2 and 3).

## 4. Discussion

We have clearly demonstrated the importance of *Shigella* and rotavirus in childhood enteric illness in the highlands of Papua New Guinea. These results support our recently published findings in the same setting investigating the prevalence and epidemiology of rotavirus in children,<sup>6</sup> and the high prevalence of *Shigella* detected by culture in adults and children seeking outpatient medical assistance.<sup>9</sup> The findings are also consistent with global trends, with a recent multicentre case-controlled study finding *Shigella*, rotavirus, and ETEC to be commonly associated with moderate to severe childhood diarrhoea.<sup>10</sup>

We did not speciate *Shigella* in this study; however, our recent study in adults and children showed *Shigella flexneri* to be the most commonly isolated species.<sup>9</sup> Other studies in this setting have shown that antimicrobial resistance in *Shigella* strains is a major concern, with multidrug resistance detected in the majority of samples.<sup>8,9</sup> The World Health Organization now recommends ciprofloxacin as the drug of choice for all patients with dysentery;<sup>11</sup> to date resistance to ciprofloxacin has not been reported in Papua New Guinea.

In this study we did not type rotavirus, however the globally circulating strains G1P[8], G3P[8], and G2P[4] were recently demonstrated to be the most prevalent in this setting.<sup>6</sup> Routine rotavirus vaccination has not been initiated in Papua New Guinea, so pre-existing immunity due to vaccination is unlikely to have influenced the incidence of rotaviral disease.

Enteric parasites were not targeted, but it is likely that pathogens such as *Cryptosporidium spp* and *Giardia spp* play an important role in gastrointestinal illness in children in this setting. A study by Phuanukoonnon and colleagues<sup>12</sup> found *Giardia* to be common in pregnant women in the highlands of Papua New Guinea; so transmission to children would seem likely. In other low-income settings *Cryptosporidium spp* are important pathogens, as demonstrated in the recent case-controlled multicentre study.<sup>10</sup> Further parasitological investigations are warranted in this setting,

**Table 1**  
Primers used for the real-time PCR/RT-PCR detection of enteric pathogens

Pathogen	Primer/probe	Sequence	Reference
Rotavirus	ROTF1	ACCATCTTCACGTAACCCCTC	27
	ROTF2	ACCATCTACACATGACCCTC	
	ROTR	CACATAACGCCCTATAGCC	
Norovirus G1	ROTP	FAM-ATGAGCACAAATAGTAAAAGCTAACACTGTCAA-BHQ1	27
	NOG1F	CGYTGATGCGNNTTYCATGA	
	NOG1R	CCTTAGACGCCATCATCATTAC	
	NORG1P	VIC-TYCGRTCTCTGTCCA-MGBNFQ	
Norovirus G2	NOG2F	CARGARBCNATGTTYAGRTGGATGAG	27
	NOG2R	TCGACGCCATCTTCATTACA	
	NOG2P	VIC-AGATYGGGATCSCCCTC-MGBNFQ	
Astrovirus	ASTF	TCTYATAGACCCGYATTATTGG	27
	ASTR	TCAAATTCTACATCATCACCAA	
	ASTP	Cy5-CCCADCCATCATCATCTTCATCA-BHQ3	
Sapovirus	SAPF	CAGGCTCTCGCCACCTAC	27
	SAPR	CCCTCCATYCAACACTAWTTT	
	SAPP	FAM-TGGTTCATAGGTGGTRC-MGBNFQ	
Adenovirus (40/41)	AdF40–41	TTCCAGCATAAATACTCWGGCTTTG	28
	AdR40–41	AATTTTTTCTGWGTCAGGCTGG	
	advMGB	FAM-CCWTACCCCTTATTGG-MGBNFQ	
Shigella spp	ShigF	ACCATGCTCGCAGAGAACT	29
	ShigR	TACGCTTCAGTACAGCATGC	
	ShigP	HEX-TGGCGTGTCCGGAGTACAGC-BHQ1	
Salmonella spp	SAompF	CCTGGCAGCGGTGATCC	30
	SAompR	AAATTTCTGCTGCGTTTGGC	
	SAompP	FAM-TGCCCTGCTGGCTGCTGCA-MGBNFQ	
Campylobacter spp	CampF	CTGCTTAACACAAGTTGAGTAGG	31
	CampR	TTCTTAGGTACCGTCAGAA	
	CampP	FAM-TGTCATCTCCACGGCGTTGCTGC-BHQ1	
Vibrio cholerae	VCctxF	TTTGTAGGCACGATGATGGAT	32
	VCctxR	ACCAGACAATATAGTTTGACCCACTAAG	
	VCctxP	FAM-TGTTTCCACCTCAATTAGTTGAGAAGTGCCB-BHQ1	
EPEC ( <i>eae</i> gene)	EAE-F	ACTGGACTTCTTATRCCGTTCTATG	33
	EAE-R1	CTAAGCGGTTATTGTTACCAGA	
	EAE-R2	CCTAAACGGGTATTATCACCAGA	
EPEC (EAF plasmid)	EAE-P	TxRd-AATCCTGATCAATGAAGACGTTATAGCCCA-BHQ2	33
	EPS-F	GTTCITGGCGAACAGGCTTGTC	
	EPS-R	TTAAGCCAGTACCATCCACCC	
ETEC (ST)	EPS-P	Cy5-AGTACTGACGTGACGGCTCGCTGTTCC-BHQ3	33
	ST-F	CTGGTTTTGATTCAAATGTTCTGTG	
	ST-R	TCCTGAGGAAAGGTGAAAAAGAC	
ETEC (LT)	ST-P	TxRd-TTGATTTCTCATATTACTCCGGACATGGCA-BHQ2	33
	LT-F	AGCGGCGCAACATTTTCAG	
	LT-R	TTGGTCTCGGTGAGATATGTGATTC	
	LT-P	FAM-TCGAAGTCCCGGCGAGTCAACATATAGA-BHQ1	

EPEC, enteropathogenic *Escherichia coli*; EAF, EPEC adherence factor; ETEC, enterotoxigenic *Escherichia coli*.

particularly given that parasitic gastrointestinal infections may have impacts beyond enteric illness (e.g. childhood development).

Although this was not a case–control study, our investigation provides important data on the presence of enteric pathogens in children presenting with diarrhoeal illness. A previous case–control study by Howard et al.<sup>5</sup> detected rotavirus in 23% (odds ratio 18.2) and *Shigella* in 13% (odds ratio 9.6) of children hospitalized with diarrhoea. On this basis, and the established knowledge that these are globally important enteric pathogens, we are confident that these pathogens are the major contributors to childhood diarrhoeal illness in this setting. The detection of *Shigella* at a higher rate than rotavirus and all other pathogens in the present study is noteworthy, particularly as children with blood or mucous in their stools were omitted from the study. Acute watery diarrhoea is commonly reported as the initial phase of shigellosis, preceding dysentery, and is often the only symptom during mild infections.<sup>13</sup> In Papua New Guinea, *Shigella* seems to be very prevalent in the community and dysentery outbreaks are frequently reported. The importance of *Shigella* in Papua New Guinea is exemplified by a recent outbreak of *Shigella* in an internally displaced population, which spread to the surrounding community and resulted in approximately 1200 cases of shigellosis and 5 deaths.<sup>34</sup>

Mixed infections were detected at a high frequency in this study (22.1%), which is comparable to the level of co-infections detected

in a diarrhoeal aetiology study conducted in Tanzania (20.7%)<sup>14</sup> and consistent with the high level of co-infections detected in other developing settings such as Jordan (15.5%),<sup>15</sup> Libya (13.8%),<sup>16</sup> Vietnam (13.5%),<sup>17</sup> and Brazil (11.0%).<sup>18</sup> In comparison, co-infections are typically reported at lower frequency in developed settings, e.g. Italy (9.8%),<sup>19</sup> Denmark (1.9%),<sup>20</sup> and France (1.1%).<sup>21</sup> These findings may be associated with increased levels of enteric pathogens in the environment due to poor levels of sanitation and hygiene in developing settings.

A recent study estimated the incidence of paediatric diarrhoea in 140 countries.<sup>1</sup> The study indicated that Papua New Guinea had the highest incidence of paediatric diarrhoea in the Western Pacific Region for all age groups (0–5, 6–11, 12–23, and 24–59 months). These figures may be a reflection of the poor access to safe water sources and improved sanitation (approximately 40% and 45% coverage, respectively) throughout the country.<sup>22</sup> Interventions to target all enteric illness are urgently required in Papua New Guinea and other developing settings, and improved sanitation and hygiene would greatly contribute to decreasing the burden of these illnesses.<sup>23,24</sup> Further interventions such as educational campaigns to improve hygiene, hand-washing with soap, and exclusive breastfeeding in young infants should also be considered, as they have been shown to significantly reduce enteric illnesses in children.<sup>25,26</sup> Moreover, further study and targeting of specific

**Table 2**

The frequency of pathogen detection in children (aged <5 years) hospitalized with acute gastroenteritis

Characteristics	Total (n=199)
Tested positive for a pathogen	138 (69.8%)
Multiple pathogens detected	44 (22.1%)
Shigella spp	53 (26.6%)
Rotavirus	51 (25.6%)
Adenovirus 40/41	23 (11.6%)
Enterotoxigenic <i>E. coli</i> (ETEC)	22 (11.1%)
Enteropathogenic <i>E. coli</i> (EPEC)	17 (8.5%)
Norovirus G2	12 (6.0%)
<i>Campylobacter</i> spp	8 (4.0%)
Norovirus G1	7 (3.5%) <sup>b</sup>
Sapovirus	4 (2.0%) <sup>b</sup>
<i>Salmonella</i> spp	2 (1.0%) <sup>b</sup>
<i>Vibrio cholerae</i>	0 (0%)
Astrovirus	0 (0%)
Co-infections <sup>a</sup>	
Shigella sp and rotavirus	10 (5.0%)
Shigella sp and ETEC	8 (4.0%)
Rotavirus and adenovirus 40/41	5 (2.5%)
Rotavirus and norovirus G1	3 (1.5%)
Norovirus G2 and EPEC	3 (1.5%)
Shigella sp and EPEC	3 (1.5%)
Shigella sp and adenovirus 40/41	3 (1.5%)
Shigella sp and <i>Campylobacter</i> spp	2 (1.0%)
Shigella sp and norovirus G2	2 (1.0%)
Rotavirus and <i>Campylobacter</i> spp	2 (1.0%)
Rotavirus and ETEC	2 (1.0%)

<sup>a</sup> Only co-infections detected in multiple patients are listed; pathogen associations also include instances where more than two pathogens were detected in a sample.

<sup>b</sup> These pathogens were detected at levels below our statistical limit of detection.

**Table 3**

Total number of co-infections for specific pathogens

Pathogen	Number of co-infections (% <sup>a</sup> )
Shigella spp	26 (49.1)
Rotavirus	22 (43.1)
Enterotoxigenic <i>E. coli</i>	11 (50.0)
Adenovirus 40/41	10 (43.5)
Enteropathogenic <i>E. coli</i>	7 (41.2)
Norovirus G2	6 (50.0)
Norovirus G1	4 (57.1)
<i>Campylobacter</i> spp	4 (50.0)
<i>Salmonella</i> spp	1 (50.0)
Sapovirus	1 (25.0)

<sup>a</sup> The proportion of occasions that a pathogen was associated with a co-infection.

pathogens such as Shigella and rotavirus, and determining the interactions between major and lesser enteric pathogens, may also be warranted in the future.

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samples were collected following informed consent from parents or guardians.

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