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Isolation of *Vibrio cholerae* and other enteric microbiota from patients with suspected cholera during the 2009 outbreak in Madang Province, Papua New Guinea

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SUMMARY

When cholera was first detected in Papua New Guinea (PNG) in mid-2009, national diagnostic capacity faced many challenges. This was in part due to the non-endemic status of the outbreak, resulting in few local staff experienced in *Vibrio cholerae* detection and poor access to the required consumables. The PNG Institute of Medical Research conducted culture on specimens from suspected cholera patients in Madang Province, with presumptive *V. cholerae* isolates sent to Goroka for confirmation. Of 98 samples analysed 15 were culture positive, with *V. cholerae* detected by polymerase chain reaction (PCR) in an additional 3 samples. Further analyses were conducted to identify other pathogenic bacteria from thiosulphate citrate bile salt sucrose (TCBS) agar. Molecular-based assays detected enteropathogenic (n = 1) and enterotoxigenic (n = 1) strains of *Escherichia coli*. No other major enteric pathogens were detected. The low detection rate of *V. cholerae* at the provincial level reflects challenges in the laboratory diagnosis of cholera and in-country challenges in responding to an outbreak of a non-endemic disease, such as lack of in-country diagnostic expertise and available consumables in the early stages. It also suggests that full aetiological investigations are warranted in future outbreaks of acute watery diarrhoea in PNG to fully elucidate the potentially complex aetiology, which could in turn guide diagnostic, treatment and prevention measures.

Introduction

The cholera outbreak in Papua New Guinea (PNG) commenced in mid-2009 in Morobe Province (1). From there the disease spread to most of Morobe, Madang and East Sepik Provinces and the Southern Region, which included National Capital District and Central, Gulf and Western Provinces. During the late stages of the outbreak, approximately 2 years after the index case, an estimated 15,500 cases and almost 500 deaths had been recorded, with ongoing transmission in the Autonomous Region of Bougainville at the time of the report (2).

Madang was the second province to be affected by the outbreak. Modilon General Hospital (MGH) recorded more than 550 cases and one death (index case). However, for the whole province 1819 cases and 40 deaths were reported late in the outbreak (2). Many patients with suspected cholera presented to the MGH in Madang, the provincial capital. In response to the outbreak, a cholera treatment centre was established on the grounds of MGH to treat patients with suspected cholera. Patients were treated based on clinical signs and symptoms, largely in the absence of supportive laboratory evidence. This is consistent with Good Clinical Practice and

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consistent with recommendations of leading global health authorities (3,4); indeed, the World Health Organization specifically states, "Don't wait for laboratory results to start treatment" (5). Timely commencement of treatment is imperative as cholera can be rapidly fatal. However, this does not negate the need for accurate diagnosis, and more specifically the absence of culture as a diagnostic tool results in a dearth of epidemiological data and antimicrobial resistance trends (6). The Port Moresby General Hospital pathology laboratory was established as the National Cholera Reference Laboratory shortly after the cholera outbreak commenced in Morobe Province. To assist with cholera diagnosis, staff from the Papua New Guinea Institute of Medical Research (PNGIMR) conducted bacterial culture and also assisted with conducting and interpreting rapid diagnostic tests. Here, we report on the findings of the bacterial culture during the Madang outbreak, including a limited investigation to seek other potential causes of acute watery diarrhoea (AWD) during the cholera outbreak.

Materials and Methods

Specimen collection for the diagnosis of cholera was conducted as part of Good Clinical Practice. Approval to conduct further analysis of bacterial isolates was given by the PNG Institute of Medical Research Institutional Review Board and the PNG Medical Research Advisory Committee (#10.54). During the peak of the cholera outbreak in Madang (October-November 2009) specimens from patients with suspected cholera (>5 years of age with AWD) were collected. Samples were taken to the PNGIMR laboratory at MGH and streaked generously on thiosulphate citrate bile salt sucrose (TCBS) agar (ThermoFisher, Australia) and incubated for 24 hours at 37°C. Profuse growth of sucrose-fermenting colonies (yellow colonies on TCBS agar) was presumptively identified as *V. cholerae*. Sucrose-fermenting colonies were taken from TCBS agar and preserved in 1.8 ml skim milk glucose glycerol broth (SGGB) and stored at -70°C. These isolates were then transported to the Bacteriology Laboratory of PNGIMR in Goroka for further identification using standard bacteriological techniques (7). Initial culture was conducted on 5% horse blood agar (BA) and MacConkey agar (MA) (ThermoFisher, Australia) and incubated overnight at 37°C. Where overnight plates had multiple colony morphologies from a single sample, each different colony type was subcultured on to BA again for purity and incubated as above. A Gram stain was conducted on all morphotypes. All Gram-negative isolates were streaked on to TCBS agar and incubated at 37°C overnight. Sucrose-fermenting colonies were further characterized using oxidase, catalase, Gram stain and sensitivity to O129 vibrio-static discs (10 µg and 150 µg) (7-9). Presumptive *V. cholerae* isolates were confirmed by polymerase chain reaction (PCR) to detect the cholera toxin gene (*ctxA*) and haemolysin gene (*hlyA*), using previously described primers (10,11). The presence of both genes is indicative of toxigenic *V. cholerae*. The thermal cycling parameters used for both target genes were: 50°C for 2 minutes, then 95°C for 15 minutes, 45 cycles of 94°C for 60 seconds and 60°C for 1 minute.

Molecular (PCR) detection of *V. cholerae* directly from the SGGB (sent from Madang to the Goroka laboratory) was conducted to determine whether some isolates had lost viability. A 200 µl aliquot of all 98 samples had a commercial deoxyribonucleic acid (DNA) extraction conducted using a DNA Mini kit and corresponding protocol (Qiagen, Australia). Extracts were preserved at -20°C for PCR detection as outlined above.

Non-*V.cholerae* isolates were identified to determine whether any were potential enteric pathogens. Initial identification of isolates was conducted using standard bacteriological methods (7). Gram-negative isolates were grouped based on the results of biochemical tests and representative isolates were characterized using the BD (Becton Dickinson) BBL Crystal Enteric/Nonfermenter Identification Kit (Becton Dickinson, USA).

Presumptive *Escherichia coli* were tested to determine whether they were verotoxigenic (VTEC), enteropathogenic (EPEC) or enterotoxigenic (ETEC) strains of *E. coli* by detection of relevant genes using loop-mediated isothermal amplification (LAMP). DNA extracts of SGGB were used (as described above). The VTEC detection kit, EPEC detection kit and ETEC detection kit (Eiken Chemical Co, Ltd, Japan) were used according to the manufacturer's instructions.

Gram-positive cocci that were oxidase and catalase negative were considered to be *Enterococcus* spp., part of the normal gut flora and not known to be enteric pathogens. Gram-

positive cocci that were oxidase negative and catalase positive were considered to be *Staphylococcus* spp. (or closely related genera). Presumptive staphylococci had a DNase test conducted: DNase-positive isolates were then tested for coagulase activity (7) using human plasma. DNase-positive, coagulase-positive isolates were considered to be *Staphylococcus aureus*. Confirmed *S. aureus* were tested for toxin production using the 3M Tecra™ Staph Enterotoxin Visual Immunoassay (VIA) Detection Kit (3M Pty Ltd, Australia) following the manufacturer's instructions.

Results

Of 134 stool samples that grew on TCBS agar, 98 were viable and sent to PNGIMR Goroka for *V. cholerae* confirmation and further testing. Of these, 15 (15%) yielded isolates with biochemical profiles consistent with *V. cholerae*, and were confirmed as toxigenic *V. cholerae* using PCR (all 15 isolates were positive for *ctxA* and *hlyA* genes). By using direct PCR detection of *V. cholerae* from SGGB (n = 98) (to detect non-viable *V. cholerae)* an additional 3 samples were found to be positive. Thus, using a combination of culture and PCR, 18 of 98 samples (18%) were confirmed to be *V. cholerae*. A summary of these results is shown in Figure 1.

Some samples yielded multiple isolates, resulting in 121 viable (non-*V. cholerae*) isolates. Of these, 61 were identified as Enterobacteriaceae, with 34 confirmed as *E. coli* using traditional bacteriological methods. With the use of LAMP, one *E. coli* isolate was positive for EPEC, one positive for ETEC, and none positive for VTEC.

14 non-lactose-fermenting Enterobacteriaceae were characterized to determine whether they were recognized enteric pathogens such as *Shigella* spp. or *Salmonella* spp. Despite sharing many biochemical traits, none was confirmed as *Shigella* spp. or

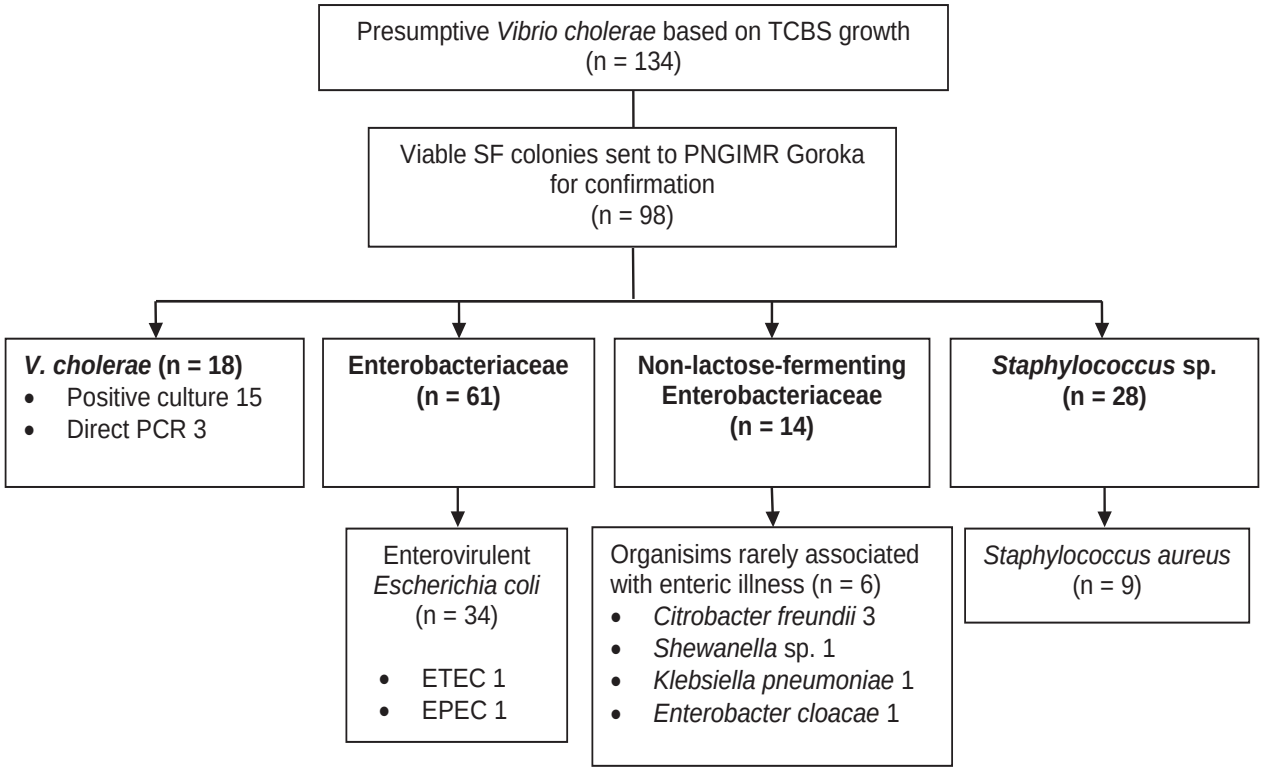

Figure 1. Flow diagram of sample processing and pathogen identification for suspected cholera patients in Madang, 2009. TCBS = thiosulphate citrate bile salt sucrose agar; SF = sucrose-fermenting; PNGIMR = Papua New Guinea Institute of Medical Research; PCR = polymerase chain reaction; ETEC = enterotoxigenic *E. coli*; EPEC = enteropathogenic *E. coli*.

Salmonella spp. 3 suspected *Salmonella* spp. were confirmed as *Citrobacter freundii*. Other Enterobacteriaceae isolates were confirmed as *Shewanella* sp., *Klebsiella pneumoniae* and *Enterobacter cloacae*.

A total of 28 isolates were preliminarily identified as *Staphylococcus* spp., with 9 confirmed as *S. aureus*. Confirmed *S. aureus* isolates were tested for toxin production; however, all were negative.

Discussion

In our study, 18% of samples from patients from Madang Province with suspected cholera were confirmed as cholera positive by the culture or PCR detection of the microbe in preserved cultures. This low rate of detection reflects both the limitation of culture as the gold standard of diagnosis for cholera, and also the challenges in infectious disease diagnosis in regional PNG.

The 15% culture-positive rate was considerably lower than the isolation rate (49%) of *V. cholerae* from suspected cholera patients at Port Moresby General Hospital pathology laboratory (6). The need to preserve and transport isolates to Goroka might be associated with a loss of viability, although detection by a culture-independent method (PCR) failed to significantly increase the detection rate (18% detection rate by combined culture and PCR). It is unlikely that the detection rate in this study is a true reflection of the proportion of people with cholera, and challenges associated with cholera diagnosis have been recently reported. Alam et al. (12) demonstrated that *V. cholerae* was the predominant cause of an outbreak of AWD; however, more than 40% of the cholera cases (confirmed by culture-independent methods) were culture negative. Two possible explanations given by Alam et al. for the high rate of culture-negative cases were the impact of lytic bacteriophages on *V. cholerae* and the ability of *V. cholerae* to quickly enter the viable but non-culturable state. Both of these mechanisms are well studied and understood in environmental conditions, but their role in vitro is not fully elucidated. Given the findings of Alam and colleagues, it is conceivable that many of the patients in Madang presenting with AWD had cholera, but the bacteria were not detected.

Enrichment broth was not used in the isolation of *V. cholerae* in Madang, and this may have led to a slight decrease in culture-positive results. However, according to the Centers for Disease Control and Prevention (8), alkaline peptone water (APW) enrichment is an optional step in the diagnosis of cholera, as patients with cholera gravis usually shed high numbers of the causative bacterium.

At the time of the outbreak in PNG it is possible that people who had diarrhoea exhibited increased health-care-seeking behaviour, in part due to educational campaigns and potentially through fear of developing more severe symptoms than they were feeling at the time of presentation. Thus patients presenting during the cholera outbreak may have had other aetiological agents causing diarrhoea, as the burden of enteric diseases in PNG is high, largely as a result of poor sanitation and hygiene (13).

The presumption that other pathogens may have played a role in cases of diarrhoea and AWD at the time of the cholera outbreak is supported by the findings of our study, despite the severe limitation of the study in this regard. The primary objective of the Madang-based culture was to confirm *V. cholerae* as the cause of AWD in a large cohort of patients, and to obtain isolates for epidemiological studies (2) and antibiotic resistance (to be reported separately). This, in combination with the lack of resources available during the cholera outbreak in Madang, led to culture being conducted primarily on TCBS agar (which is inhibitory to many other bacterial enteric pathogens) and sucrose-fermenting organisms growing on TCBS being sent to IMR Goroka for confirmation. Despite this limitation, we were able to detect other enteric pathogens and emerging/opportunistic pathogens. Both EPEC and ETEC were detected. ETEC produces heat-labile toxins, which can induce powerful bowel purging with rice-water stools, similar to symptoms of cholera (14). ETEC has been linked to cholera-like outbreaks in Asia and South America in the past (15,16). EPEC has also caused watery diarrhoea. Cholera-like diarrhoea has also been attributed to *Salmonella* sp, (17), a common enteric pathogen that has been detected in foods in PNG in the past (18), though not commonly isolated in the Madang region (ML, personal communication). No *Salmonella* were detected in this study, perhaps in part due to the apparent low prevalence in this

region, but more likely due to the limitations of the study design. Potential and opportunistic pathogens were isolated from a small number of samples, namely *Citrobacter freundii* (n = 3), *Shewanella* sp. (n = 1), *Enterobacter cloacae* (n = 1) and *Klebsiella pneumoniae* (n = 1). All of these organisms have on occasion been linked to enteric illness (19-22), although they are not considered major diarrhoeal pathogens.

In response to the cholera outbreak in PNG, culture services were re-established in Lae (complementing existing services in Port Moresby), but not in other provincial capitals where cholera occurred. Relevant government and non-government organizations were reluctant to supply laboratories outside Port Moresby and Lae with the required consumables for *V. cholerae* culture, in part due to the supply chain difficulties faced in PNG. The preliminary identification conducted in Madang had little impact on clinical management as all patients were treated syndromically. However, the rapid presumptive identification may have assisted in mobilizing a response; and results were used in daily cholera briefings. Moreover, the confirmation and subsequent analysis of Madang isolates at the better equipped Goroka laboratory was of epidemiological value (2, unpublished data).

Infectious disease diagnosis remains problematic in PNG, particularly in regional areas. The cholera outbreak highlighted these problems, and strategies should be developed in an attempt to strengthen diagnostic capacity in key regional centres. Rapid diagnostic tests (RDTs) may play a future role in diagnostics, particularly in outbreak situations. Recent studies demonstrated the potential utility of RDTs for diagnosis of important febrile illnesses in PNG (23,24), and a potential role for cholera RDTs in PNG has been suggested in the light of challenges associated with culture-based diagnostics (6). However, RDTs are not a diagnostic panacea, and this was highlighted in the Madang outbreak where the first 18 RDTs conducted were interpreted as negative (LM and ML, personal communication). In light of the subsequent confirmation of the cholera outbreak, it is possible the RDTs were being misinterpreted. Training needs to accompany the introduction of new diagnostic methods, and there is a risk that this will be overlooked or will be found logistically challenging during a disease outbreak.

Other diagnostic methods should also be explored. With nucleic acid detection methods becoming more robust and user-friendly, their application in low-income settings is becoming more viable. Kirkham and colleagues stated that PCR-based diagnostics could complement traditional diagnostic methods for pneumonia and meningitis in PNG (25), and this may similarly apply to other infectious diseases, particularly in reference laboratories.

Conclusion

V. cholerae was detected in 18% of specimens in this study, a lower proportion than expected during a confirmed outbreak of cholera. As found in other settings, the causative organism may not always be detected in true cholera cases. However, our detection rate was lower than reported in other studies, and our findings highlight the challenges associated with in-country diagnosis of outbreak diseases and reflect shortcomings in regional laboratory capacity in PNG. If better patient health outcomes are to be achieved for infectious diseases in general, there is a need for practical diagnostic methods, complementary to culture, that are suitable for use in low-income settings. Rapid diagnostic methods may serve this purpose, with the potential to be applicable in outbreak situations.

During the initial cholera outbreak in PNG, the focus was on confirming cases of cholera and implementing disease control measures. However, we have demonstrated the presence of other pathogens in patients with AWD, despite the limitations in study design that impeded our ability to detect non-*Vibrio* pathogens. In future outbreaks a subset of samples should be comprehensively analysed using a variety of diagnostic methods to enable a better understanding of the contribution of other enteric pathogens to the aetiology of AWD during cholera epidemics. Different aetiological agents may justify different treatment and control strategies. In particular, efforts to detect ETEC are warranted given the role it has played in AWD outbreaks in other regions.

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REFERENCES

1 **Rosewell A, Dagina R, Murhekar M, Ropa B, Posanai E, Dutta SR, Jennison A, Smith H, Mola G, Zwi A, MacIntyre CR.** *Vibrio cholerae* O1 in 2 coastal villages, Papua New Guinea. *Emerg Infect Dis* 2011;17:154-156.

2 **Horwood PF, Collins D, Jonduo MH, Rosewell A, Dutta SR, Dagina R, Ropa B, Siba PM, Greenhill AR.** Clonal origins of *Vibrio cholerae* O1 El Tor strains, Papua New Guinea, 2009-2011. *Emerg Infect Dis* 2011;17:2063-2065.

3 **Carter J, Müller-Stöver I, Östensen H, Heuck CC.** Good clinical diagnostic practice: a guide for clinicians in developing countries to the clinical diagnosis of disease and to making proper use of clinical diagnostic services. Cairo: World Health Organization Regional Office for the Eastern Mediterranean, 2005.

4 **Bauernfeind A, Croisier A, Fesselet JF, van Herp M, Le Saoût E, McCluskey J, Tuynman W.** Cholera Guidelines 2004. Second edition. Paris: Médecins Sans Frontières, 2004.

5 **World Health Organization Global Task Force on Cholera Control.** First steps for managing an outbreak of acute diarrhea. Geneva: World Health Organization, 2010.

6 **Greenhill AR, Rosewell A, Kas M, Manning L, Latorre L, Siba PM, Horwood PF.** Improved laboratory capacity is required to respond better to future cholera outbreaks in Papua New Guinea. *Western Pac Surveill Response J* 2012;3:30-32.

7 **Barrow GI, Feltham RKA, eds.** Cowan and Steel's Manual for the Identification of Medical Bacteria. Third edition. Cambridge: Cambridge University Press, 1993.

8 **Bopp CA, Ries AA, Wells JG.** Isolation and identification of *Vibrio cholerae* serogroups O1 and O139. In: McIntyre L, ed. Laboratory Methods for the Diagnosis of Epidemic Dysentery and Cholera. Atlanta: National Centre for Infectious Disease (NCID), Centers for Disease Control and Prevention (CDC), in cooperation with World Health Organization Regional Office of Africa, 1999:41-54.

9 **McLaughlin JC.** Vibrio. In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH, eds. Manual of Clinical Microbiology. Sixth edition. Washington: ASM Press, 1995:465-476.

10 **Blackstone GM, Nordstrom JL, Bowen MD, Meyer RF, Imbro P, DePaola A.** Use of a real time PCR assay for detection of the ctxA gene of *Vibrio cholerae* in an environmental survey of Mobile Bay. *J Microbiol Methods* 2007;68:254-259.

11 **Lyon WJ.** TaqMan PCR for detection of *Vibrio cholerae* O1, O139, non-O1, and non-O139 in pure cultures, raw oysters, and synthetic seawater. *Appl Environ Microbiol* 2001;67:4685-4693.

12 **Alam M, Hasan NA, Sultana M, Nair GB, Sadique A, Faruque AS, Endtz H, Sack RB, Huq A, Colwell RR, Izumiya H, Morita M, Watanabe H, Cravioto A.** Diagnostic limitations to accurate diagnosis of cholera. *J Clin Microbiol* 2010;48:3918-3922.

13 **Horwood PF, Greenhill AR.** Cholera in Papua New Guinea and the importance of safe water sources and sanitation. *Western Pac Surveill Response J* 2012;3:3-5.

14 **Qadri F, Svennerholm AM, Faruque AS, Sack RB.** Enterotoxigenic *Escherichia coli* in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. *Clin Microbiol Rev* 2005;18:465-483.

15 **Sack RB, Gorbach SL, Banwell JG, Jacobs B, Chatterjee BD, Mitra RC.** Enterotoxigenic *Escherichia coli* isolated from patients with severe cholera-like disease. *J Infect Dis* 1971;123:378-385.

16 **Vicente AC, Teixeira LF, Iniguez-Rojas L, Luna MG, Silva L, Andrade JR, Guth BE.** Outbreaks of cholera-like diarrhoea caused by enterotoxigenic *Escherichia coli* in the Brazilian Amazon Rainforest. *Trans R Soc Trop Med Hyg* 2005;99:669-674.

17 **Saidi SM, Yamasaki S, Lijima Y, Kariuku S.** Cholera-like diarrhoea due to *Salmonella* infection. *J Infect Dev Ctries* 2011;5:68-70.

18 **Greenhill AR, Shipton WA, Omoloso AD, Amoa B, Warner JM.** Bacterial contamination of sago starch in Papua New Guinea. *J Food Prot* 2007;70:2868-2872.

19 **Guarino A, Capano G, Malamisura B, Alessio M, Guandalini S, Rubino A.** Production *of Escherichia coli* STa-like heat stable enterotoxin by *Citrobacter freundii* isolated from humans. *J Clin Microbiol* 1987;25:110-114.

20 **Guerin F, Le Bouguenec C, Gilquin J, Haddad F, Goldstein FW.** Bloody diarrhea caused by *Klebsiella pneumoniae*: a new mechanism of bacterial virulence? *Clin Infect Dis* 1998;27:648-649.

21 **Karawasa T, Ito H, Tsukamo T, Yamasaki S, Kurazono H, Faruque SM, Nair GB, Nishibuchi M, Takeda Y.** Cloning and characterization of genes encoding homologues of B subunits of cholera toxin and *Escherichia coli* heat-labile enterotoxin from clinical isolates of *Citrobacter freundii* and *E. coli*. *Infect Immun* 2002;70:7153-7155.

22 **Nath R, Saikia L, Chowdhury G, Das PP.** Isolation of *Shewanella algae* from rectal swabs of patients with bloody diarrhoea. *Indian J Med Microbiol* 2011;29:422-425.

23 **Manning L, Laman M, Rosanas-Urgell A, Turlach B, Aipit S, Bona C, Warrell J, Siba P, Mueller I, Davis TM.** Rapid antigen detection tests for malaria diagnosis in severely ill Papua New Guinean children: a comparative study using Bayesian latent class models. *PloS One* 2012;7:e48701.

24 **Siba V, Horwood PF, Vanuga K, Wapling J, Sehuko R, Siba PM, Greenhill AR.** Evaluation of serological diagnostic tests for typhoid fever in Papua New Guinea using a composite reference standard. *Clin Vaccine Immunol* 2012;19:1833-1837.

25 **Kirkham LA, Smith-Vaughan HC, Greenhill AR.** Improving aetiological diagnosis of bacterial pneumonia and meningitis in Papua New Guinea. *PNG Med J* 2010;53:139-146.