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Molecular Screening of Major Bacterial Enteropathogens in Human Stool Samples from Diarrhoeal Outbreak Sites

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ABSTRACT

Background: *V. cholera* types have been implicated often in a number of occasions in diarrhoeal outbreaks in Nepal. The recent outbreak in Far Western Nepal, 2009, was also attributed primarily to *V. cholera*. Molecular tools were used for the first time on some of the samples from the outbreak to screen for major pathogens present in those samples.

Methods: A commercial multiplex PCR kit based bacterial enteropathogen screening assessment was carried out on 33 human stool samples from areas of a diarrhoeal outbreak in Nepal. A total of 10 pathogenic bacterial strains at the genus level were targeted using primers provided by the manufacturer.

Results: Bacterial pathogens were detected in 23 samples (69.7%). Vibrio species was detected at an overall frequency of 36.4% followed by Aeromonas spp (33.3%) and Shigella spp (15.2%) along with Verocytotoxin producing E. coli (VTEC) family (15.2%). The frequency of singly occurring pathogen in all samples was 18.2% with most of the bacterial pathogens detected in combination with other pathogens at a frequency of 60.6%.

The study also shows that majority 73 (86%) of the research centers didn't start the research yet.

Conclusions: This first ever molecular screening study shows that bacterial screening is indeed possible in diarrhoeal samples. The results obtained from this study will enable monitoring of future such outbreaks using similar techniques.

Keywords: bacterial pathogens, diarrhoea, multiplex pcr, Nepal.

INTRODUCTION

Numerous bacterial and viral pathogens have been implicated in diarrhoeal outbreaks worldwide.[1] Among those, *V. cholera* types have been implicated often in a number of occasions. The first report of cholera was officially published for the years 1958 to 1960 in Kathmandu by a medical doctor visiting Nepal.[2] Other reports of cholera outbreaks in Nepal have been reported in literature.[3,4] Outbreak of acute diarrheal disease in Kavre district during the year 2005 was investigated which showed 31% cases positive for *V. cholera* 01 El Tor Ogawa associated with other parasites as well.[5] The recent 2009 outbreak of watery diarrhoea in districts of Mid- and Far West regions of Nepal affected over 5000 individuals and caused more than 200 deaths. Findings from Jajarkot district in Far West Nepal showed more than 100 people died in just 4 months in the district, with over 425 affected.[6] Recent outbreaks in the country have been found to be due partially to the *V. cholera* 01 El Tor Ogawa strain.[3,6-7] *V. cholera ctx* gene was detected using the method of Southern hybridization by a Japanese research group.[4]

To date, the detection of *V. cholera* in Nepal has relied almost extensively on microbiological, biochemical and serological tests.[3,6] This study was designed as a preliminary screening assessment, limited to genus level identification of the major bacterial pathogens normally implicated in a diarrhoeal outbreak situation.

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METHODS

A descriptive study design which includes molecular screening of microorganisms in stool samples from mid and far western region of Nepal during diarrheal disease outbreak. This was a part of outbreak investigation, hence ethical approval was not possible to obtain but confidentiality was maintained throughout the study.

A total of 61 stool samples were collected from at least two different diarrhoeal outbreak affected districts in the Mid and Far Western region of Nepal for genomic based screening. Among those, 11 samples were collected from Mid-Western region while 50 samples were collected from Far-Western region of Nepal. Cary Blair transport medium (Hi Media) with swabs were used for the collection of stool samples. Those were transported to Nepal Public Health Laboratory, Kathmandu for primarily *V. cholera* identification purposes using microbiological and serological methods. Some of the sample swabs were re-inoculated into their original Cary Blair transport containers and transported to Centre for Molecular Dynamics Nepal (CMDN) affiliated laboratory for genomic based pathogen screening. Not all samples were received with swabs in place due to various reasons, and thus could not be utilized for the genomic assessments.

Among the samples obtained, a total of 33 samples (11 from Mid-Western region of Nepal and 22 randomly selected samples from Far-Western region of affected area) were used in this study. Samples were processed in four main steps that included Incubation, Pre-treatment, DNA Extraction and Multiplex PCR. Selected swabs were immersed in 2 ml of 0.8% NaCl and incubated overnight at 37°C. Incubated samples were processed further to obtain bacterial pellets re-suspended in 200µl Phosphate Buffered Saline (PBS) followed by DNA extraction and purification using Qiagen DNeasy Blood and Tissue kit. Obtained DNA was eluted in 60µl of elution buffer and further processed for Multiplex PCRs.

Commercial screening kits (Seeplex® Diarrhoea ACE Detection kit) were used for the screening test. A multiplex PCR assay for detecting a number of bacterial pathogenic strains was performed as per manufacturer's instructions. The bacterial panels included: Diarrhea B1 ACE detection - *Salmonella* spp. (*S. bongori and S. enterica*), *Shigella* spp.(*S. flexneri, S. boydii, S. sonnei and S. dysenteriae)*, *Vibrio* spp.(*V. cholerae, V. parahemolyticus and V. vulnificus)*, *Campylobacter* spp.(*C. jejuni and C. coli)*, and *Clostridium difficile*; Diarrhea B2 ACE Detection - *Yersinia enterocolitica*, *Aeromonas* spp. (*A. salmonicida, A. sobria, A. bivalvium, A. hydrophila)*, *E.coli* O157:H7, VTEC family, and *Clostridium perfringens*.

Briefly, a volume of 3µl of extracted DNA was mixed with 17µl of Master mix and subjected to PCR amplification cycle. Amplification was performed on PTC-225 Peltier thermal cycler (MJ research) under the following cycling conditions: 15 min of initial denaturation at 94°C, followed by 40 cycles of 30 sec denaturation at 94°C, 90 sec of annealing at 60°C and 90 sec of extension at 72°C. At the end of 40 cycles, a 10 min extension was performed at 72°C. Amplified products were analysed using gel electrophoresis in 2.0% agarose gel with 0.5µg/ml Ethidium bromide and run in 1X TBE buffer. DNA bands were observed under UltraViolet light of 260nm wavelength and digitally recorded.

RESULTS

The multiplex PCR screening of 33 samples used in this study for major genes associated with known diarrhoeal bacterial pathogens showed that overall, 23(69.7%) samples returned positive results. Among positive samples (n=23), there were 11(47.8 %) samples which were observed to harbor at least one strain of bacteria. In the rest of the samples (n=12; 52.1%), pathogens were observed to occur in groups of two or more. Apart from samples showing absence of infecting pathogens (n=10), the majority of samples appeared to harbour single pathogens (n=11). Furthermore, at least 10 samples were observed to harbour more than one type of pathogen from the panel of pathogens screened. Two samples were positive for 3 and 5 different pathogens, respectively. The frequency of pathogen detection in overall samples (Table 2). The number of samples positive for any one pathogenic strain (n=11) was closely followed by those positive for a combination of any two pathogens (n=10). One sample tested positive for 3 different pathogenic strains while another one sample returned positive results for five different pathogenic strains. Among the samples showing presence of only one pathogen, *Vibrio* species was observed in highest frequency. The pathogenic strains that were observed to occur singly (other pathogens were not detected) (Table 3).

The diarrheal pathogens identified in this study, based on their unique Genus specific genes, were *Vibrio* species, *Aeromonas* spp, *Campylobacter* spp, *Shigella* spp, VTEC family, and *E. coli* O157:H7. Among 33 samples, *Vibrio* species was the dominant pathogen occurring in 12(36.4%) samples followed by *Aeromonas* spp which occurred in 11(33.3%) samples. *Shigella* spp was detected in 15.2% of samples tested while *Campylobacter* spp was detected in 12.1% of the samples. Pathogens under the family of Verotoxin Producing *E. coli* (VTEC) were also detected in 5(15.2%) samples while *E. coli* 0157:H7 were detected in 2(6.1%) samples. The total percentage of various pathogens detected in this study (Table 3). *Yersinia enterocolitica*, *Salmonella* spp, *Clostridium difficile* and *Clostridium perfringens* were not detected in any of the samples.

The two most frequent diarrheal bacterial pathogens that were detected were *Vibrio* spp and *Aeromonas* spp. In both cases, the co-infecting bacterial pathogens detected were *Shigella* spp. (33.3% and 22.2% with *Vibrio* spp and *Aeromonas* spp respectively) and *Campylobacter* spp (22.2% in both cases respectively). The other major co-infective bacterial pathogens in both cases were *E. coli* O157:H7 and VTEC family respectively, although their numbers were low. Figure 1 and Figure 2 depict the percentage of bacterial pathogens in association with *Vibrio* spp and *Aeromonas* spp respectively.

Table 1. List of pathogens (species in brackets) and the corresponding target gene(s).

Pathogen	Target gene (as per manufacturer information)
Vibrio spp. (V. cholera, V. parahemolyticus, V. vulnificus)	hly, tlh, wh
C. difficile Toxin B	tcdB
Salmonella spp. (S. bongori , S. enterica)	sopB
Shigella spp. (S. flexneri, S. boydii, S. sonnei, S. dysenteriae)	vif, ipaH
Campylobacter spp. (C. jejuni, C. coli)	hip, asp
C. perfringens	cpa, cpe
Y. enterocolica	inv
E. coli O157:H7	rfb, fliC
VTEC	VT1, VT2
Aeromonas spp. (A. salmonicida, A. sobria, A. bivalvium, A. hydrophila)	hly, ela

hly- hemolysin gene; *tlh*-Thermilabile hemolysin; *sopB*: *Salmonella* Outer Protein B; *ipaH* invasin plasmid Antigen; *hip*- Hippuricase; *asp*-aspartyl Kinase gene; *cpa-Clostridium perfringens* alpha toxin gene; *cpe-Clostridium perfringens* enterotoxin gene; *tcdB*- Toxin B gene of *Clostridium difficile*; *inv*- invasin gene; *rfb*-surface antigen(somatic O-antigen) cluster; *fliC*-Flageller antigen(H-gene) cluster; *VT1*, *VT2*- Verocytotoxin 1 and 2.

Table 2. Pathogens detected using multiplex PCR method of screening 33 diarrheal stool samples.

Bacterial strain	Frequency of Detection	Percentage (%) frequency
Vibrio spp	12	36.4
Aeromonas spp	11	33.3
Shigella spp	5	15.2
VTEC	5	15.2
Campylobacter spp	4	12.1
E.coli O157:H7	2	6.1

Table 3. Frequency of detection of organism.

Organism	Frequency of detection	Percentage of samples
Vibrio spp	4	12.1
Aeromonas spp	3	9.1
VTEC	2	6.1
Shigella spp	1	3.0
Campylobacteria spp	1	3.0
TOTAL	11	33.3

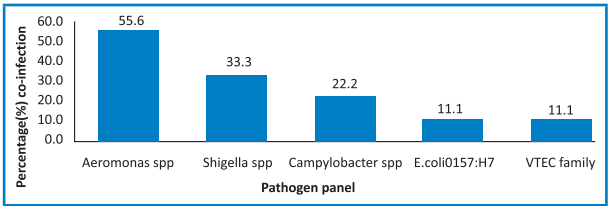

Figure 1. Graph depicting the overall percentage of bacterial pathogens co-detected with *Vibrio* spp.

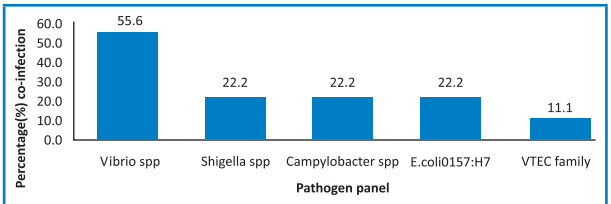

Figure 2. Graph depicting the overall percentage of co-detection of bacterial pathogens with *Aeromonas spp*.

DISCUSSION

Detection of microorganisms using microbiological and serological methods continues to be practiced in Nepal.[8,9] Although molecular methods such as Southern hybridisation method have been used for identifying genes of some bacterial strains detected in stool samples from outbreak areas in Nepal, the research work actually took place outside Nepal.[4] Previous studies have indicated that *V. cholera* 01 subtype is the major cause for diarrhoeal outbreaks in different parts of Nepal in the past.[5,6] However, presence of other pathogenic bacterial strains in such outbreaks is not well assessed. There have been no studies to date that have tried to screen for presence of other major pathogens during an outbreak situation in Nepal.

The reason for non-detection of pathogen in 10 samples among the 33 samples assessed in this study could be attributed to presence of different strains of the bacterial group other than those targeted using the commercial kit. Also, errors in sampling method leading to acquisition of less than adequate amount of stool

cannot be ruled out either. The sampling of stool using Cary Blair medium was carried out by recently trained health assistants in remote areas of Nepal, and human errors can be considered a possibility. The results from this study have shown that *Vibrio* species was present in a high percentage in the samples that were screened. The majority of the *Vibrio* pathogens found in an outbreak have been reported to be strains of *V. cholera*.[5,6,9,10] Our *Vibrio* spp result correlates with that reported for *V. cholera* 01 strain detection for a different outbreak area in Nepal during the same time frame.[5] This particular outbreak thus might be attributed primarily to *Vibrio cholera*. However, the interesting finding from this study is that there were a number of other important pathogens associated with *Vibrio* spp in a majority of cases. For example, *Aeromonas* spp., usually associated with contaminated aquatic environments[11,12] was the major co-infecting pathogen in the samples tested. The family of Verocytotoxic *E. coli* (VTEC) was also detected in a large number of samples that were identified as being *Vibrio* spp. positive. VTEC comprise of a number of *E. coli* strains implicated in life-threatening diarrhoeal diseases.[13-15] These findings suggest that the outbreak in Mid- and Far- West Nepal was primarily due to contaminated water, which the population consumed without treating it to rid it of pathogens. Majority of the pathogens detected here at a high frequency rate are water-borne diarrheal pathogens.[1] There was a high presence of *Aeromonas* spp., which is primarily water-borne organism but is known to cause cholera-like diarrhoeal symptoms.[16] The role of this pathogen in diarrhoea has been established in literature.[1] This in itself is a reason for concern in medical treatment of diarrhoea as *Aeromonas* spp. and *Vibrio* spp. require different antibiotics at different dosages. Furthermore, there were suspicions that food was also a contributing factor in this outbreak.[17] However, the nature of the pathogens observed in this study does not appear to support this claim for the regions the samples came from as the pathogens identified are mostly those implicated in water-borne diseases. *Campylobacter* strains are sometimes associated with food poisoning in diarrhoeal cases but the detection of this pathogen was low compared to those implicated in water-borne diarrhoeal cases.[18-24] *Salmonella* spp is known to be mostly associated with food stuff and in some cases with contaminated drinking water.[25]

This is the first ever genomic screening assessment of samples from a diarrheal outbreak carried out entirely in Nepal. The results have provided new insights into the type of bacterial pathogens involved, and the combinations in which they occur. This information is expected to assist in future outbreak investigations in the current endemic diarrheal outbreak areas of Nepal. Furthermore, this may enable proper treatment regimes to be formulated for such outbreaks.

CONCLUSIONS

This study showed that *Vibrio* species appeared to be the major causative agent in the 2009 diarrhoeal outbreak in selected areas from which samples were accessed. However, there were number of other pathogens such as *Aeromonas spp*. that were also observed in those samples. The use of molecular methods such as PCR facilitated the screening of those pathogens as a rapid method of pathogen detection. This method of screening is very useful to detect microorganisms at the beginning for the successful prevention and control of any outbreak.

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