Multidrug resistant *Vibrio cholerae* O1 and detection of *ctx* gene from clinical and environmental specimens in Kathmandu valley

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**ABSTRACT**

**Background:** Cholera, an infectious disease caused by *Vibrio cholerae*, is a major public health problem particularly burden in some of the developing countries including Nepal. Although the recent outbreaks of cholera are due to *V. cholerae* El Tor all around the world, the classical biotypes are still predominant in Nepal. Serogroup O1 of *V. cholerae* classical biotype was the primary cause of cholera outbreak in Kathmandu in 2012. In the study, we reported the toxigenic strains of *V. cholerae* from both environmental and clinical specimens by detecting the *ctx* gene.
Results: Among twenty four *V. cholerae* isolates, 91.7% were clinical and 8.3% were from water samples. All isolates were serogroups O1 of *V. cholerae* classical biotype and subserotype, Ogawa. All isolates were resistance to ampicillin, nalidixic acid and cotrimoxazole; followed by 90.9% resistance to erythromycin. However, tetracycline was found to be the most effective drug for the isolates. All those isolates were multidrug resistance and possessed *ctx* gene of approximately 400 base pairs indicating the toxigenic strains.

Conclusion: The studies of the last fifteen years showed rapid emergence of multidrug resistant *V. cholerae* isolates. Moreover in the study, we found 100% MDR strains. So, it is suggested on the identification of toxigenic strains and proper antibiotic susceptibility test for effective empirical therapy to control the possible threats by *V. cholerae*.

Key words: *Vibrio cholerae* O1 Classical, Resistant profile, Multidrug resistance, Cholera toxin (*ctx*) gene

BACKGROUND

Cholera is the second leading cause of mortality worldwide among children under 5 years, and is one of the main causes of morbidity in adults (Bryce et al., 2005; Harris et al., 2012). The causative agent of cholera, *Vibrio cholerae* is a genetically versatile bacterial species (Alam et al., 2014). More than two hundred serogroups were identified on the basis of the somatic O antigens (Chatterjee and Chaudhuri, 2003) among which O1 and O139 are two major virulent strains. Two biotypes of *V. cholerae* O1; classical and El Tor are the causative agents of the sixth and the seventh pandemics respectively (Ramamurthy and Nair, 2010). Organisms of both biotypes of serogroup *V. cholerae* O1 are further subdivided into Serotypes; Inaba, Ogawa and Hikojima ((Kaper et al., 1995)). *V. cholerae* O1 is still frequently isolated from many outbreak regions of Asian countries (Yi et al., 2014) including Kathmandu, Nepal (Pun et al., 2012) mainly because of inadequate sanitation and poor access to safe drinking water.

*V. cholerae* usually spreads by the fecal-oral route by ingesting fecally contaminated water or food, person to person transmission and direct contact with infected feces (Tandukar et al., 2008). The antimicrobial therapy is commonly recommended for shortening duration, reduction of symptomatology and bacterial excretion. However the emergence of multidrug resistant *V.
*V. cholerae* isolates is a major cause of problem in developing countries today (Vila and Pal, 2010) because of an inappropriate use of antimicrobial agents (Thomson and Sterky, 1986).

The major virulence factors of cholera are mainly associated with the CTX genetic element corresponds to CTX Φ (prophage), a lysogenic filamentous bacteriophage. The genetic element comprises of two gene clusters, the core and the RS2 regions. The core region contains *ctx* genes encoding cholera toxin (CT) and five more genes encoding the required components for phage morphogenesis (Yi et al., 2014). The toxin thus produced is transported extracellularly by type II secretion system disrupting ion transport of intestinal epithelial cells (Sanchez and Holmgren, 2011). The subsequent loss of water and electrolytes leads to severe secretory diarrhea, a characteristic of cholera (Maheshwori et al., 2011). The presence of such genes confirms the toxigenic strains of *V. cholerae*. Thus, the study screened for the presence of *ctx* gene in all clinical and environmental isolates for identification of virulent strains and rapid diagnosis of cholera.

**METHODS**

**Informed consent:** The written consent from the participants' involvement in the research were obtained for the study according to the standard protocol formulated by Nepal Health Research Council (NHRC), Nepal.

**Isolation, Identification and typing of *V. cholerae***: A total of 450 stool samples from diarrheal patients and 30 water samples from the cholera outbreaks regions in Kathmandu valley were collected and processed to isolate and identify *V. cholerae* following the standard laboratory methods (Forbes et al., 2007). The isolates were subjected for serotyping using kit (Mast Group and Denka Seiken, Japan) as per kit's instruction. The biotyping was carried out by using Polymyxin B sensitivity test, Voges Proskauer reaction and chicken RBC agglutination tests (Kaper et al., 1995). The research was ethically approved by Nepal Health Research Council (NHRC), Kathmandu, Nepal.
**Antibiotic Susceptibility Test:** The antimicrobial susceptibility test of the isolates to various antimicrobial disks (Ampicillin-10mcg, Nalidixic acid-30mcg, Ciprofloxacin-5mcg, Cotrimoxazole1.25/23.75mcg, Cefotaxime-30mcg, Chloramphenicol-30mcg, Tetracycline-30mcg and Erythromycin-15mcg) was done by modified Kirby-Bauer disk diffusion method as recommended by Clinical and Laboratory Standards Institute guidelines (CLSI, 2012). *Escherichia coli* (ATCC, 25922) was used for the standardization of the Kirby-Bauer test for correct interpretation of the zone diameters.

**Molecular Assay:** The genomic DNA of all isolates were extracted and purified from the aerobically grown culture in Luria Bertani (LB) broth and used for specific PCR for the detection of *ctx* genes (Ranjbar *et al.*, 2008). A pair of primers; C2F (5’-AGGTGTAAAATTCCTTGACGA-3’) and C2R (5’-TCCTCAGGGTATCCTTCATC-3’) were used for the gene amplifications as described by ta and Lin, 1995. Briefly the reaction mixture for the gene amplification was prepared in 25 µl consisting of 12.5 µl Qiagen mastermix, 1 µl 10 µM forward primer, 1 µl 10 µM reverse primer, 9.5 µl distilled water and 1.0 µl of template DNA. The amplifications were performed as follows: an initial pre-denaturation at 94°C for 15 min followed by 35 cycles at 94°C for 30 sec (denaturation), 60°C for 60 sec (primer annealing), 72°C for 60 sec (DNA extension) and a final elongation was performed at 72°C for 10 min on a thermocycler (Thermal cycler Perkin Elmer cetus P11966). The amplified products were fractionated by electrophoresis through 1.5% agarose gel with NEB 100 bp marker DNA which was visualized by staining the gel with ethidium bromide (Patrick *et al.*, 2012).

**Data analysis:** Data were entered and analyzed using SPSS software for Windows (version 21).

**RESULTS**
Altogether twenty four *V. cholerae* were isolated out of which 91.7% were from diarrheal patients and 8.3% were from water samples. Among clinical isolates, 50% were isolated from adult patients of 20-29 years.

**Serotyping and Biotyping of *V. cholerae***
All strains were found to be serogroup O1, serotype Ogawa and the Classical biotypes (Table 1).

**Antibiotic resistance pattern**
All clinical *V. cholerae* strains were susceptible to Tetracycline. However, susceptibility to ciprofloxacin and Chloramphenicol were 90.9% and Cefotaxime were 81.8%. While all isolates were found to be resistance to Ampicillin, Nalidixic acid and Cotrimoxazole and 90.9% isolates were resistance to Erythromycin.

Among environmental *V. cholerae* isolates, all were resistance to Ampicillin, Nalidixic acid and Cotrimoxazole. However, 50% isolates were resistance to Chloramphenicol and Erythromycin.

**Antibiotic resistance profile**

Five different types of resistant profiles were observed among clinical isolates. All isolates were CR$_1$ resistant type followed 68.2% isolates in CR$_2$ type. Similarly, the resistant types, CR$_3$, CR$_4$ and CR$_5$ were seemed in 13.6%, 4.5% and 4.5% isolates respectively (Table 2).

Among environmental isolates, 3 different profiles were observed. Fifty percent isolates showed ER$_2$ profile and 50% were ER$_3$ type (Table 2).

**Multidrug resistant *V. cholerae* and Detection of ctx gene**

All the isolates were found to be multidrug resistance (Table 2) and highly pathogenic strains possessing ctx gene of approximately 400 base pairs (Figure 1).

**DISCUSSION**

Cholera is found to be one of the most predominant diarrheal diseases in Nepal even these days. In the study, we found 4.9% cholera cases among diarrheal patients and 6.67% water samples were positive for *V. cholerae*. Similar study by Karki and Tiwari in Kathmandu reported 25.1% cholera cases in 2004 (Karki and Tiwari, 2007). The study by Tamang *et al.* in Kavre also reported 31% positive cases for *V. cholerae* in the same year (Tamang *et al.*, 2005). The frequencies of *V. cholerae* among diarrheal patients were found to be still higher (Kanskar *et al.*, 2011, Karki *et al.*, 2012 and Shah *et al.*, 2012). The higher rate of the pathogens in the studies might be due to the hospital based analysis, however the prevalence could be low among the community based studies. The predisposing factors such as poor sanitation, lack of safe drinking water, unhygienic foods stuffs might be responsible for repeated occurrence of the pathogens in Kathmandu and some more districts of Nepal.

*Vibrio cholerae* El Tor O1 Ogawa had been responsible for the endemic in Nepal before 2012 (Shah *et al.*, 2012; Tamang *et al.*, 2005) and previous outbreaks of cholera in Kathmandu valley
in 2004 (Malla et al., 2005). In contrary, all the isolates in the study were *V. cholerae* O1 of serogroups Ogawa and classical biotype. Infections with classical strains are generally more severe than those with El Tor strains (Kaper et al., 1995). Three strains; *V. cholerae* O1 biotype El Tor, *V. cholerae* O1 biotype Classical and *V. cholerae* O139 were frequently isolated in cholera outbreaks in Asian countries (Faruque et al., 1998)). Although classical *V. cholerae* O1 caused the fifth and sixth pandemics, and presumably the earlier pandemics, the seventh pandemic was attributed to the El Tor biotype, which has now again replaced by the classical biotype in the study. Inaba and Hikojima sero subtypes were not found in the study. Different researches conducted in Nepal had reported the occurrence of both Ogawa and Inaba serotypes with an interval of several years (Pokharel et al., 2009; Sherchand et al., 2009; Shrestha, et al., 2008; Tamang et al., 2005).

Children and the elderly people are mostly affected by cholera (Harris et al., 2012; Rai and Bhatta, 2004; Taneja et al., 2004). Contrary to this, adult populations of age group 20-29 years were highly infected in our context. The studies by Kansakar et al., 2011 and Yadav et al., 2012 found similar results in which most of the infected patients were adult population of the age group 20 to 29 years and 15 to 29 years respectively. The more infections in these groups may be due to their food habits outside the home and consumption of street foods.

All the strains in the study were resistance to nalidixic acid, cotrimoxazole and ampicillin suggesting no role of these drugs in the treatment of cholera. Das et al., (2011) and Tran et al., (2012) also reported 100% resistance to the above three antibiotics. A high incidence of cotrimoxazole resistance *V. cholerae* O1 strains has been reported in the studies in Africa, Asia and South America (Goel and Jiang, 2010; Ibarra and Alvarado, 2007; Mandomando et al., 2007). The study by Karki and Tiwari (2007) found that all the *V. cholerae* strains were resistance to ampicillin while 97.8% isolates were susceptible to ciprofloxacin. Generally, fluoroquinolone have excellent activity against *V. cholerae*, but fluoroquinolone resistant strains of *V. cholerae* have lately been reported from India (Das et al., 2011; Garg et al., 2001; Mukhopadhyay 1998). The majority of the *V. cholerae* strains in the study were susceptible to tetracycline (100%), ciprofloxacin (90.9%), cefotaxime (81.8%) and chloramphenicol (90.9%) which might be effective alternative drugs for the treatment, though further development of resistance needs to be monitored. A similar result was also found by Shah et al., 2012 showing sensitivity of 90% and 77.3% to cefotaxime and chloramphenicol respectively. However they
showed 81.8% resistant strains to tetracycline. Garg et al., (2000) reported high-level resistance to chloramphenicol in India. This result was just contrast to our findings. Contrary to macrolide resistance rarely being reported in the studies by Harris et al., 2012 and Kansakar et al., 2011, a high level erythromycin resistance (90.9%) was found in the present study. Resistance to erythromycin and other antimicrobial agents among V. cholerae can be acquired through selected mutations over the time, or due to widespread use of antibiotics for prophylaxis in asymptomatic individuals (Sharifi-Mood and Metanat, 2014)

All V. cholerae were found to be multidrug resistance in the study. MDR cholera epidemics have been reported from countries; Bangladesh (Shah et al., 2006), Pakistan (Siddiqui et al., 2006) and Nepal (Karki et al., 2012, Pun et al., 2012). Indiscriminate use of antibiotics in the treatment of cholera and other enteric diseases led emergence of antibiotic resistance among V. cholerae. MDR V. cholerae with epidemic outbreaks (both classical and El Tor biotypes) had been reported worldwide (Mandal et al., 2011). Multiple drug resistance in V. cholerae can be attributed to either spontaneous mutation or to the horizontal transfer of resistance genes between members of gut coliform or other co-existing microflora and Vibrio (Speer et al., 1992).

All tested V. cholerae strains possessed ctx gene in our study. Toxigenic strains of Vibrio cholerae possessed essential genetic element called the CTX genetic element (Juliana et al., 2000; Chen et al., 2004). The isolates in the study were thus confirmed as toxigenic strains. The ctx genes are located in the CTX element and encode the cholera toxin CT. This toxin is primary responsible for the severe secretory diarrhea in infected person. Thus we screened all the isolates for the presence of ctx gene. Our results showed the presence of ctx gene of approximately 400 bp in all the tested strains similar as described by Patrick et al., 2012. Similar genes were also detected in the environmental isolates. Chakraborty et al., 2000 also found the critical virulence genes in the environmental strains of V. cholerae.

**CONCLUSIONS**

V. cholerae and many other types of organisms are the causative agents of diarrhea in human. Among these pathogens, V. cholerae is the one primary strains isolated in the epidemic diarrheal disease, cholera. However not all V. cholerae are toxigenic and epidemic. So, it is suggested for
regular examination of *V. cholerae* for the presence of *ctx* gene from clinical and non-clinical samples to ensure the toxigenic strain and proper antibiotic susceptibility test such pathogens that support the prevention of the possible outbreaks of cholera.

**ABBREVIATIONS**

AST  Antibiotic Susceptibility test  
ATCC  American Type Culture Collection  
CLSI  Clinical Laboratory Standard Institutes  
CR  Resistant profile of Clinical *V. cholerae* isolates  
CT  Cholera Toxin  
*ctx*  Cholera toxin producing gene  
DNA  Deoxyribonucleic Acid  
ER  Resistant profile of Environmental *V. cholerae* isolates  
KCMS  Kantipur College of Medical Sciences  
KTM  Kathmandu  
LB  Luria Bertani  
MDR  Multi Drug Resistance  
NHRC  Nepal Health Research Council  
RLABB  Research Laboratory for Biotechnology and Biochemistry  
UGC  University Grants Commission  
PCR  Polymerase Chain Reaction
COMPETING INTERESTS

The authors declare that they have no competing interest.

AUTHORS' CONTRIBUTIONS

"Mr. Upendra Thapa Shrestha developed this proposal with help of Mr. Nabaraj Adhikari. He brought financial support from University Grant Commission, Nepal and did the molecular part of the research. Nabaraj Adhikari participated in proposal development and molecular work of the research. Rojina Shrestha worked on hospital for isolating and identifying *V. cholerae* from clinical samples among diarrheal patients and obtained ethical approval for the work from Nepal Health Research Council (NHRC), Nepal. Dr. Megh Raj Banjara worked on data analysis part of the research and contributed in manuscript writing. Mr. Komal Raj Rijal worked on isolation of identification of *V. cholerae* from environmental specimens; drinking water samples from Kathmandu valley. Prof. Dr. Shital Raj Basnyat participated in all microbiological works in identifying and typing of *V. cholerae*. Prof. Dr. Vishwanath Prasad Agrawal participated in all molecular biology works of the research. All authors read and approved the final manuscript.”

ACKNOWLEDGMENT

The authors are heartily thankful to University Grant Commission (UGC), Sanothimi, Bhaktapur, Nepal for financial support to this work as well all the participants in the research.

REFERENCES


15. CLSI- Clinical Laboratory Standard Institute: Performance standards for antimicrobial susceptibility testing; twenty second informational supplement, 32:M100-S22, 2012.

17. Shangkuan YH, Lin HC. Application of Random


28. Shrestha


Figure 1: Amplification of ctx gene in V. cholerae isolates
Table 1: Serotyping and Biotyping of *V. cholerae*

<table>
<thead>
<tr>
<th>Typing methods</th>
<th>Tests performed</th>
<th>Serotypes</th>
<th>No of positive strains (%)</th>
<th>Types</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serotyping</td>
<td>Agglutination (Mast Group and Denka Seiken Kit, Japan)</td>
<td>Ogawa, Inaba, Hikojima</td>
<td>24 (100)</td>
<td>Serotypes Ogawa</td>
</tr>
<tr>
<td>Biotyping</td>
<td>Voges Proskauer Polymyxin B sensitivity Chicken cell agglutination</td>
<td>24 (100) Sensitive (100)</td>
<td>0</td>
<td>Classical biotypes</td>
</tr>
</tbody>
</table>

Table 2: Antibiotic resistant profile of *V. cholerae* isolates

<table>
<thead>
<tr>
<th>Resistant types</th>
<th>Resistant profile</th>
<th>No. of isolates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical isolates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR₁</td>
<td>Ampicillin, Nalidixic acid, Cotrimoxazole</td>
<td>22 (100)</td>
</tr>
<tr>
<td>CR₂</td>
<td>Ampicillin, Nalidixic acid, Cotrimoxazole, Erythromycin</td>
<td>15 (68.2)</td>
</tr>
<tr>
<td>CR₃</td>
<td>Ampicillin, Nalidixic acid, Cotrimoxazole, Erythromycin, Cefotaxime</td>
<td>3 (13.6)</td>
</tr>
<tr>
<td>CR₄</td>
<td>Ampicillin, Nalidixic acid, Cotrimoxazole, Erythromycin, Chloramphenicol, Ciprofloxacin</td>
<td>1 (4.5)</td>
</tr>
<tr>
<td>CR₄</td>
<td>Ampicillin, Nalidixic acid, Cotrimoxazole, Erythromycin, Chloramphenicol, Ciprofloxacin, Cefotaxime</td>
<td>1 (4.5)</td>
</tr>
<tr>
<td>Environmental isolates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ER₁</td>
<td>Ampicillin, Nalidixic acid, Cotrimoxazole</td>
<td>2 (100)</td>
</tr>
<tr>
<td>ER₂</td>
<td>Ampicillin, Nalidixic acid, Cotrimoxazole, Erythromycin</td>
<td>1(50)</td>
</tr>
<tr>
<td>ER₃</td>
<td>Ampicillin, Nalidixic acid, Cotrimoxazole, Erythromycin, Chloramphenicol</td>
<td>1(50)</td>
</tr>
</tbody>
</table>

*Note: CR-Clinical resistance type, ER- Environmental resistance type*