Antibiogram and Minimum Inhibitory Concentration of selected antibiotics against *Vibrio cholerae* Isolated from Sewage Samples

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ABSTRACT

Objectives: The purpose of this study was to perform antibiogram profiling and determine minimum inhibitory concentration (MIC) of selected antibiotics against *Vibrio cholerae* isolated from sewage samples.

Methods: A cross-sectional study was carried out at the microbiology and molecular biology laboratory of Central Campus of Technology. A total of 45 sewage samples were collected. The samples for this study was aseptically collected from different places of Sunsari district of Eastern Nepal and subjected to isolation and identification of *V. cholerae*., followed by antibiotic susceptibility testing by Kirby Bauer disc diffusion method. MIC was performed by selecting three antibiotics ampicillin, tetracycline and streptomycin and was done by microtiter plate method.

Results: A total of 24 *V. cholerae* (53.33%) were isolated. From the total samples, 53.33% (n=13) were found to be resistant against Erythromycin and Tetracycline. 66.67% (n=16) samples showed that the bacterium was resistant to cefotaxime. 93.33% (n=22;) samples were sensitive to ciprofloxacin where as 66.67% (n=16) samples were sensitive to ampicillin and cotrimoxazole. 14 isolates were inhibited by Ampicillin at concentrations below 8 µg/ml, while 8 and 10 isolates were inhibited by Tetracycline and Streptomycin, respectively at concentrations below 4 µg/ml.

Conclusions: This sewage-based *V. cholerae* antibiogram study underscores sewage as a reservoir and urges sewage control. ampicillin, erythromycin, and tetracycline show limited efficacy, in contrast to ciprofloxacin, ampicillin, and cotrimoxazole, which remain viable for treating much of cholera.

Keywords: *Vibrio cholerae*, antibiotic resistance, minimum inhibitory concentration, Sewage.

INTRODUCTION

Cholera is a sudden-onset diarrheal illness caused by the bacterium *Vibrio cholerae*, a Gram-negative rod from the Vibrionaceae family. There are around 200 different serogroups of *V. cholerae* identified based on their O antigen. Among these, only the O1 and O139 serogroups are known to cause cholera, while the others, non-O1 and non-O139 groups, have minimal public health impact (Momba & Azab El-Liethy, 2017).

Sewage contamination, primarily due to human fecal matter, plays a significant role in spreading waterborne diseases like cholera. This contamination is particularly dangerous for children, who are more vulnerable to these illnesses. Globally, waterborne diseases such as acute watery diarrhea, dysentery, hepatitis, and typhoid affect an estimated 3.4 million people annually, with cholera being a significant contributor (Liu et al., 2016).

Date of Submission: September 22, 2024
Date of Acceptance: November 18, 2024
Published Online: December 31, 2024
DOI: https://doi.org/10.3126/tujm.v11i1.85495

Cholera has a long history in Nepal, with the first recorded outbreak occurring in 1823. The country has faced several cholera outbreaks since then, including the ongoing seventh cholera pandemic, which began in South Asia in 1961 (Rhee *et al*., 2020). Nepal experiences endemic cholera, leading to the deaths of 30,000 to 40,000 people each year due to diarrheal diseases, with cholera being a major cause. Notable outbreaks include the Jajarkot incident in 2009, which affected 3,000 people and resulted in over 80 deaths, and the Nepalgunj outbreak in 2010, which impacted 1,500 people and caused eight deaths. These outbreaks were primarily due to poor sanitation practices and contaminated water sources (Gupta et al., 2016).

Before 1970, antibiotics were effective in treating cholera and diarrhea caused by *V. cholerae*. However, over the past five decades, the bacterium has developed significant antibiotic resistance. The emergence of tetracycline resistance was first noted during a major cholera epidemic in Tanzania in 1977-78 (Towner *et al*., 1979). This resistance had serious public health consequences, as seen in the death of 12,000 people in Goma, Eastern Zaire, due to tetracycline-resistant *V. cholerae* strains. The newly emerged O139 serogroup in 1993 also showed resistance to tetracycline. Starting from the early 1990s, *V. cholerae* developed resistance to multiple antibiotics, including ampicillin, nalidixic acid, chloramphenicol, and tetracycline, expanding the spectrum of resistance. Today, *V. cholerae* strains show resistance to nearly all antibiotics commonly used for treatment, presenting a serious challenge to public health (Das et al., 2020).

Antibiotic resistance in *V. cholerae* is a growing global health concern. In Nepal, the earliest study on *V. cholerae* resistance was conducted by in 1996, which reported reduced sensitivity to nalidixic acid, co-trimoxazole, ampicillin, and cephalexin (Ise T, 1996). Subsequent studies found complete co-trimoxazole resistance and significant resistance to other antibiotics. By 2008, cholera isolates from various outbreaks in Nepal exhibited complete resistance to furazolidone, co-trimoxazole, and nalidixic acid. A comprehensive study between 2007 and 2010 involving 522 isolates from ten hospitals in Nepal found widespread resistance: 100% to nalidixic acid and furazolidone, 90% to co-trimoxazole, 21% to tetracycline, 16% to erythromycin, and 4% to ciprofloxacin. Multi-drug resistance was observed in 6.5% of 116 stool specimens in a 2012 study (Shakya et al., 2012).

Further studies revealed that *V. cholerae* strains in Nepal have shown rapid and erratic shifts in antimicrobial susceptibility. For instance, ampicillin resistance fluctuated from 93% in 2006 to 18% in 2010, then back to 100% by 2016. Cotrimoxazole resistance also varied between 76% and 100%, while resistance to ciprofloxacin, initially absent, emerged in 2007, peaked in 2012, and declined to zero by 2016. Tetracycline resistance, which was initially low, spiked in 2007 before decreasing again. Similarly, furazolidone resistance decreased from 100% in 2006 to 5% in 2016. Although ceftriaxone resistance was initially absent, partial susceptibility emerged in 2013, with one isolate showing resistance by 2016 (Rijal et al., 2019).

This study emphasizes the presence of drug-resistant *V. cholerae* strains in sewage samples, highlighting the role of sewage in transmitting the bacteria through contaminated food and water. Analyzing these resistance patterns provides insight into the evolution of antibiotic resistance and helps identify effective treatment options for cholera.

METHODS

Study design, Study site and Sample size

A laboratory based cross-sectional study was carried out on the microbiology and molecular biology lab of Central Campus of Technology. The sample used in this study was sewage. For this study, a total of 45 sewage samples were collected. The samples for this study was aseptically collected randomly from different places of Sunsari district of Eastern Nepal.

Sample collection and transportation

Sewage samples were aseptically collected from stagnant and polluted sites in Sunsari district using 100 ml sterile BOD bottles. Protective gear, including gloves and face masks, was worn during collection. The bottles were dipped into the sewage for sample collection, then carefully transferred to the laboratory. Alkaline peptone water was added to the samples to promote bacterial growth. The collected samples were placed in an insulated container with ice and promptly transported to the laboratory, minimizing any delay in processing.

Isolation of *V. cholera*

The collected sewage samples were diluted 1:10 with sterile distilled water to obtain a usable concentration of organisms. To isolate *V. cholerae*, thiosulfate-citrate-bile-

salt-sucrose (TCBS) (Himedia, India) agar was prepared, autoclaved at 121°C for 15 minutes, and then poured into sterile petri plates to cool and solidify. For each sample, 1 ml of the diluted sample was aseptically spread across the TCBS medium using a sterile rod. The plates were incubated at 37°C for 24 hours. After incubation, green-colored colonies, indicative of *V. cholerae*, were observed and sub cultured onto Nutrient agar (Himedia, India), followed by another 24-hour incubation at 37°C. To preserve the isolated organisms, Nutrient broth (Himedia, India) medium was prepared with 10% sterile glycerol(SRL). This broth was used to maintain the viability of the isolates for extended periods (Huq et al.,2012).

Identification of organism

Various biochemical test (gram negative, catalase positive, oxidase positive, indole positive, string positive, methyl red negative, Voges-Praskauer variable, sucrose positive, motility) was performed for further confirmation (CDC, 2024).

Antibiotics susceptibility test

The modified Kirby Bauer disc diffusion method was employed to conduct the antibiotic susceptibility test. In this process, Mueller-Hinton agar was prepared and utilized as the medium onto which the bacterial sample was introduced. Following this, discs containing antibiotics were positioned onto the surface of the inoculated agar. A variety of antibiotics, including Ampicillin (AMP) (10 µg), Ciprofloxacin (CIP) (5 µg), Cotrimoxazole (COT) (25 µg), Erythromycin (E) (15 µg), Streptomycin (STR) (10 µg), Tetracycline (TET) (30 µg), Amikacin (AK) (30 µg), and Cefoxitin (CX) (30 µg), were employed in the testing process (CLSI, 2013). The plates were subsequently placed in an incubator set at a temperature of 37°C for a duration of 24 hours, during which observations were conducted (CLSI, 2013).

Minimum inhibitory concentration by broth dilution method

The microdilution method, following CLSI (2013) guidelines, was employed to determine the minimum concentrations of Ampicillin, Tetracycline, and Streptomycin for *V. cholerae*. Precise amounts of Ampicillin, Tetracycline, and Streptomycin powder were measured, and stock solutions of 256 µg/ml, 256 µg/ml, and 512 µg/ml, respectively, were prepared. A specific volume of 0.5 McFarland bacterial culture suspension was introduced to each well containing TSB broth. Using the stock solutions, various drug concentrations ranging from 128 µg/ml to 0.25 µg/ml were created in round bottom microtiter plates through a sequential dilution process. The plates also included wells for both positive and negative controls. The microtiter plates were then placed in an incubator at 37°C for 24 hours. The well with the highest drug concentration that successfully inhibited bacterial growth is considered as the MIC (CLSI, 2013).

RESULTS

Out of 45 sewage samples collected from Sunsari district, 24 (53.33%) tested positive for *Vibrio cholerae*. In Tarahara, 11 out of 15 samples showed the bacterium's presence. In Maikhola, 1 out of 3 samples tested positive. In Dharan, 12 out of 27 samples collected from various sites, including Vijaypur, Railway, and multiple wards, also tested positive for *V. cholerae*. Figure 1 shows the isolation of bacterium from different places of Sunsari district. The total isolated *V. Cholerae* is shown in comparison to total samples collected from different sites.

Of the total samples, 53.33% (13/24) were found to be resistant against Erythromycin and Tetracycline. 66.67% (16/24) samples showed that the bacterium was resistant to exposure of cefotaxime antibiotics. 93.33% (22/24) samples showed sensitive results when exposed to ciprofloxacin where as 66.67% (16/24) samples were sensitive to ampicillin and cotrimoxazole (Table 1).

Out of 24 samples, 14 were found to be inhibited by minimum concentration of Ampicillin drugs which is less than 8 µg/ml. This shows a significant association between the resistant, inhibitory and sensitive nature of bacterium to the Ampicillin ($p=0.005462$) (Table 2). Eight and ten samples were inhibited by MIC of Tetracycline ($p=0.022483$) and Streptomycin ($p=0.0034$) drugs respectively which is less than 4 µg/ml. This shows a significant association between the resistant, inhibitory and sensitive nature of bacterium to Tetracycline and Streptomycin drugs respectively (Table 3 and 4).

Table 1: Antibiotics susceptibility pattern of *Vibrio cholerae.*

Antibiotic disc	Resistant, n (%)	Intermediate, n (%)	Sensitive, n (%)
Ampicillin (10 µg)	3 (13.33%)	5 (20%)	16 (66.67%)
Amikacin (30 µg)	4 (20%)	_	20 (80%)
Cefotaxime (30 µg)	16 (66.67%)	3 (13.33%)	5 (20%)
Ciprofloxacin (5 µg)	_	2 (6.67%)	22 (93.33%)
Cotrimoxazole (25 µg)	6 (26.67%)	2 (6.67%)	16 (66.67%)
Erythromycin (15 µg)	13 (53.33%)	3 (13.33%)	8 (33.33%)
Streptomycin (10 µg)	3 (13.33%)	11 (46.67%)	10 (40%)
Tetracycline (30 µg)	13 (53.33%)	_	11 (46.67%)

Table 2: Minimum Inhibitory concentration of *Vibrio cholera* against Ampicillin

Isolates	MIC (µg/ml) of Ampicillin			P value
Vibrio cholerae	≤ 8 Sensitive	16 Intermediate	≥ 32 Resistant	0.0054
	14 (58.33%)	6 (25%)	4 (16.67%)	

Table 3: Minimum Inhibitory concentration of *Vibrio cholera* against Tetracycline

Isolates	MIC (µg/ml) of Tetracycline			P value
Vibrio cholerae	≤ 8 Sensitive	16 Intermediate	≥ 32 Resistant	0.022
	8 (33.33%)	2 (8.33%)	14 (58.33%)	

Table 4: Minimum Inhibitory concentration of *Vibrio cholera* against Streptomycin

Isolates	MIC (µg/ml) of Streptomycin			P value
Vibrio cholerae	≤ 8 Sensitive	16 Intermediate	≥ 32 Resistant	0.0034
	10 (41.67%)	8 (33.33%)	6 (25%)	

DISCUSSION

The study conducted in the Microbiology laboratory of the Central Campus of Technology, Hattisar, Dharan, focused on analyzing the prevalence and antibiotic resistance profiles of *Vibrio cholera* isolated from sewage samples collected from various locations within Sunsari District, Nepal. The research aimed to understand the extent of contamination by *V. cholera* in sewage systems and to assess the bacterium's susceptibility to commonly used antibiotics.

Vibrio are rod-shaped, gram-negative bacteria, known for their motility due to a single polar flagellum. These bacteria are widely found in estuarine and marine environments, with certain species identified as causative agents of acute gastroenteritis, wound infections, and primary septicemia in humans. Studies have shown that *Vibrio* species, including *V. cholerae*, can be isolated from both untreated sewage and treated effluent, indicating the limited effectiveness of conventional wastewater treatment processes in eliminating these pathogens (Osuolale & Okoh, 2018). This highlights the role of wastewater as a

potential vector for spreading *Vibrio* in surrounding environments, contributing to public health risks.

In this study, a total of 45 sewage samples were collected from different parts of Sunsari district, with 24 samples (53.33%) testing positive for *V. cholerae*. The sampling strategy was influenced by several factors, including the expected variations in bacterial presence across different locations, logistical challenges such as time and cost, and the study's main objectives. These considerations ensured a balanced and practical approach to sampling, enhancing the robustness of the study.

The results showed a significant prevalence of *V. cholerae* in the sewage samples, with a p-value of 0.047, indicating a meaningful relationship between the bacterium's presence and the sewage samples. Despite the varying sample sizes from different locations, the significant p-value suggests that the detection of *V. cholerae* is closely linked to specific environmental and location characteristics, which could explain the differences in prevalence across the sites. The random sampling method used across different sites ensured an unbiased representation, reinforcing the validity of the results.

Following the isolation of *V. cholerae*, the bacteria were subjected to an Antibiotic Susceptibility Test (AST) following the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI). The disc diffusion method was used to assess the bacteria's resistance to selected antibiotics, also based on CLSI recommendations. The study found that 53.33% (13/24) of the samples were resistant to Erythromycin and Tetracycline. Additionally, 66.67% (16/24) of the samples showed resistance to Cefotaxime, while 93.33% (22/24) were sensitive to Ciprofloxacin. Furthermore, 66.67% (16/24) of the samples were sensitive to Ampicillin and Cotrimoxazole.

The Minimum Inhibitory Concentration (MIC) was determined for three antibiotics: Ampicillin, Tetracycline, and Streptomycin, using the microtiter plate method. The MIC results revealed that 14 out of 24 samples were inhibited by Ampicillin at concentrations below 8 µg/ml, with a significant p-value of 0.005462, indicating a strong association between the bacteria's resistance, inhibitory, and sensitive responses to Ampicillin treatment. Similarly, 8 out of 24 samples were inhibited by Tetracycline at concentrations below 4 µg/ml (p-value 0.022483), and 10 out of 24 samples were inhibited by Streptomycin at concentrations below 4 µg/ml (p-value 0.0034), both showing significant associations with the respective treatments.

The findings of this study are consistent with previous research on *V. cholerae* prevalence and antibiotic resistance. A study in Northwest Ohio found *V. cholerae* in 5 out of 6 environmental samples, with a prevalence rate of 59.5%, similar to the 53.33% prevalence observed in this study (Daboul, 2020). It was reported a prevalence rate of 59.5% in environmental samples from India, further supporting the association between environmental factors and the presence of *V. cholera* (Mishra *et al.,* 2011).

The study also highlighted significant resistance to Ampicillin, with 13.33% (3/24) of the samples showing resistance. This result aligns with findings, who reported a 17% resistance rate to Ampicillin in *V. cholera* (Olayinka and Anthony, 2017). However, the study's findings on Tetracycline resistance, where 53% of the samples were resistant, contrast with previous study, which reported a 38% resistance rate. Notably, none of the samples in this study were resistant to Ciprofloxacin, a result that diverges from previous findings, where a small percentage of samples showed resistance to both Tetracycline and Ciprofloxacin. This discrepancy underscores the growing concern over the effectiveness of commonly used antibiotics against *V. cholerae*, especially in regions with high resistance rates. The findings suggest that alternative antibiotics with proven efficacy may be necessary in treating cholera.

The low resistance rates observed for Streptomycin in this study are consistent with findings which, further validating the potential use of this antibiotic in cholera treatment (You, 2016). The sensitivity of *V. cholerae* to Ciprofloxacin suggests that it could be an effective first-line treatment option, particularly in areas with low resistance rates. This study emphasizes the importance of regular antibiogram studies to monitor the evolving resistance patterns of *V. cholerae* and guide appropriate antibiotic selection for treatment.

The implications of this study extend beyond antibiotic resistance, highlighting the public health risks associated with sewage contamination by *V. cholerae*. The presence of drug-resistant strains in sewage can contribute to the

spread of resistance in the environment, potentially impacting the effectiveness of cholera outbreak management. Improving sewage treatment and sanitation infrastructure is crucial to prevent the spread of *V. cholerae* and mitigate the public health risks posed by inadequate water, sanitation, and hygiene practices.

Conclusion

Vibrio cholerae in sewage revealed its presence in 54.5% of 44 samples, highlighting sewage as a cholera transmission source. High resistance was noted to cefotaxime (67%) and moderate resistance to erythromycin and tetracycline (54%), while ciprofloxacin (93%) and cotrimoxazole (67%) remained effective. MIC testing further confirmed varying resistance levels, aiding in cholera treatment decisions. In conclusion, this study provides valuable insights into the prevalence and antibiotic resistance of *V. cholerae* in sewage samples from Sunsari District. The findings highlight the need for updated treatment guidelines and strategies to control the spread of cholera, emphasizing the importance of effective sewage treatment and sanitation measures in public health efforts. Regular monitoring of antibiotic resistance patterns is essential to ensure the continued effectiveness of treatments for cholera and to inform public health policies aimed at controlling the spread of this infectious disease.

ACKNOWLEDGEMENTS

We would like to thank the Department of Microbiology, Central Campus of Technology, Dharan, Tribhuvan University, Nepal, for providing laboratory facilities to complete this research work.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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