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Research article

QUANTIFICATION AND CHARACTERIZATION OF *VIBRIO CHOLERAE* ISOLATES FROM JAJU SAGAR DAM, NEEMUCH, (M.P.): A SEARCH FOR ESBL POSITIVE STRAIN

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ABSTRACT: Here we present an analysis of the occurrence, enumeration, seasonal prevalence, characterization and ESBL status of *Vibrio cholerae* strains isolated from Jaju Sagar dam (raw water), Hingoria treatment plant (treated water) and Municipal tap (tap water). Seventy two samples (24 each of raw, treated and tap) were analyzed for total viable count of V. cholerae by Direct Plating Method using Thiosulphate Citrate Bile Salt Sucrose Agar. Sampling was done twice a month covering a period of 1 year (Jan 2013-Dec 2013).Results from the current study showed 100 % occurrence of *V. cholerae* in all the raw, treated and tap water samples, throughout the year. Significant ($P<0.05$) differences were observed in *V. cholerae* log counts among Raw, Treated and Tap water. Seasonal studies of raw water samples showed highest counts ($P<0.05$) in rainy season (July-Oct 2013) followed by summer (Mar-June 2013) and winter (Nov-Feb 2013). Throughout the whole investigation period no sample of treated and tap water complied with the drinking water standards laid down by WHO and BIS. All the tap water samples contained higher counts ($P<0.05$) than the treated water samples, throughout the year. These results suggest that water treatment plants in developing countries need to be monitored to ensure they achieve WHO acceptable levels of water quality. Another important aspect of our study was to determine the antibiotic susceptibility patterns of the isolates to frame Extended Spectrum β –lactamase (ESBL) detection. The study of antibiogram on 100 isolates revealed that all the tested strains (100%) were sensitive to tetracycline, imipenem and co-trimoxazole. 60% of the *V. cholerae* isolates were resistant to aztreonam,55% to cefuroxime,40% to cephotaxime,50% to ceftazidime, 35% to ceftriaxone, 25% to amoxyclav, 95% strains were sensitive and 5% were intermediate to cefoxitin. 60 strains, that were resistant to third generation cephalosporins were selected for further detection of the ESBL by Double Disc Synergy test but all these 60 strains gave negative Double Disc Synergy test hence no ESBL positive V.cholerae was detected in the present study. Due to the lack of a genetic analysis of these strains, the exact nature of the underlying mechanisms mediating the resistance is not clear.

Key words: Antibiotic, ESBL, Hingoria treatment plant, Neemuch, Vibrio

INTRODUCTION

Despite modern techniques for disinfection, sanitation, and water purification, waterborne diseases still threaten human health. Access to safe drinking water has improved over the last decades in almost every part of the world, but approximately one billion people still lack access to safe water and over 2.5 billion lack accesses to adequate sanitation .The diarrheal disease, Cholera, which has caused epidemics and pandemics, continues to be a global threat to public health [19] .Every year millions of cholera episodes occur throughout the world especially in developing countries and thousands of cases are reported to be fatal [20]. In 2011, WHO reported 5, 89 854 cholera cases from 58 countries with death rate increased from 7543 to 7816 [50]. With the same aim in mind the present study was intended to study the occurrence and enumeration of *V .cholerae* in Jaju Sagar Dam, a potential and sole source of drinking water for all the 1,27 000 inhabitants of Neemuch. The residents of Neemuch are served by a single water treatment plant–Hingoria Treatment Plant which treats water through flocculation by Alum, Rapid Sand Filtration and finally through chlorination process. The treatment plant distributes treated water directly to the community, without any routine bacteriological testing. Hence our study also aimed to screen the possibilities of the presence and persistence of *V.cholerae* in treated and tap water to evaluate the efficiency of Hingoria treatment plant.

We also attempted to focus on the antibiogram study of *Vibrio cholerae* isolates to frame the ESBL detection. β –lactam antibiotics are widely used in the treatment of serious infections due to gram-negative bacteria. Resistance to these newer β –lactams due to β –lactamases enzymes emerged quickly. These enzymes are becoming increasingly expressed by many strains of pathogenic bacteria with the increasing potential for horizontal transfer to other strains. They compromise the activity of wide-spectrum antibiotics creating major therapeutic difficulties with a significant impact on the outcome of patients.

MATERIAL AND METHODS

Study area and Source of *Vibrio cholerae* strains: The study was conducted on Jaju Sagar Dam, a vital and sole source of drinking water to the residents of Neemuch. *V. cholerae* was isolated and enumerated from the *Dam* (raw water), *Hingoria treatment plant* (treated water) and *Municipal tap* (tap water), twice a month for a period of 1 year (Jan 2013-Dec 2013).

Isolation and Enumeration of *Vibrio cholerae*: A total of 72 samples comprising 24 samples each of raw water, treated water and tap water were examined .Enumeration of *V. cholerae* was done by direct plating method on TCBS agar, selective (Himedia, Mumbai, Maharashtra, India) [1,2].1 ml aliquot from the undiluted 100 ml sample was added to the sterile Petri dish in triplicates to which was added previously boiled and cooled (50^0C) TCBS agar. The plates were incubated at 35-37^{o}C for 18-24 hrs. After incubation, all the yellow (sucrose -fermenting) and blue-green (non-sucrose fermenting) colonies were considered total *Vibrio* colonies and counted. To differentiate *V. cholerae* from other sucrose fermenting yellow vibrio's, all the yellow colonies were sub cultured on nutrient agar without added NaCl. The *Vibrio* able to grow on 0% NaCl and giving a positive oxidase test was confirmed as *V. cholerae* [4] and counted. The CFU (colony forming units) of the three plates were counted to calculate mean cfu/ml, which was converted into log cfu/ml.

Statistical Analysis: In order to determine the significance difference between the microbiological quality of water from the Source, Treatment plant and Point of use, **one –way Analysis of Variance (ANOVA)** by means of Microsoft Excel Version 7 was employed to test the significance difference in the mean log counts of the raw, treated and tap water samples. Seasonal shifts in the raw water samples were also studied using ANOVA.

Morphological & Cultural Characteristics: Morphological characteristics were studied by using conventional microbiological techniques Viz. light microscopic observations of Gram-stained smears. Motility was tested under light microscope of 100 magnification by using slide with a drop of young bacteria and by stabbing the culture into deep tubes of mannitol motility test medium (Himedia, Mumbai, Maharashtra, India), appearance of cloudiness was evident for motility. To study cultural characteristics, colony morphology was observed on TCBS agar as well as on nutrient agar .Type of growth on nutrient agar slant and in nutrient broth was also studied. Oxygen requirement was determined by inoculation in fluid thioglycollate medium (Himedia, Mumbai, Maharashtra, India) deep tubes.

Physiological Characteristics: A series of 22 biochemical tests (Table: 2) commonly used to identify *V. cholera* [4, 5, 7, 12, 9, 17, 18, 29, 35] were used to confirm *V. cholerae*. Test cultures were grown in nutrient agar for approximately 24 hr before inoculation into test media. All the results were read after 24-48 h at 37^0C.

Antimicrobial Susceptibility Test & ESBL detection: A total of 2,986 strains were isolated throughout the year. Antibiotic sensitivity test was carried out on 100 randomly selected *V. cholerae* isolates after being confirmed through all the morphological and cultural characteristics followed by the set of 22 biochemical tests. Antimicrobial susceptibility was determined by Kirby-Bauer disc diffusion method as per the CLSI [13] in Mueller-Hinton agar plates with commercially available antibiotic discs (Himedia, Mumbai, Maharashtra, India)**.** These included tetracycline (30mcg), co-trimoxazole (25mcg), erythromycin (15mcg), amoxyclav 30mcg (amoxicillin 20mcg/clavulinic acid 10mcg), imipenem (10mcg), cefuroxime (30mcg), and cefoxitin (30 mcg). For the purpose of ESBL detection- aztreonam 30mcg (oxyimino-monobactam) and ceftazidime 30mcg, cefotaxime 30mcg, ceftriaxone 30mcg (oxyimino- cephalosporins) were used.

The isolates that were resistant to any of the third generation cephalosporin by disc diffusion method were selected for further detection of the ESBL by double disc synergy test DDST, CLSI, [14]. A disc of amoxyclav (30 mcg) was placed on the center of the Mueller-Hinton agar (Himedia, Mumbai, Maharashtra, India) plate which was previously inoculated with resistant strain. Each disc of ceftazidime (30 mcg), cefotaxime (30mcg), ceftriaxone (30mcg) and aztreonam (30mcg) was placed around the amoxyclav disc 20 mm apart to detect the synergy and incubated for 24 hr at 37^{o}C. A clear extension of the edge of the inhibition zone of any of the antibiotics towards the disc containing the clavulanate (synergy) indicates ESBL production.

RESULTS AND DISCUSSION

The objective of this study was threefold: to determine the occurrence, abundance and seasonal incidence of *V.cholerae* in Jaju Sagar Dam, secondly to evaluate the efficacy of *Hingoria treatment plant* by comparing the total viable counts (log cfu/ml) of *V .cholerae* in treated and tap water with the counts of raw water and lastly to search for ESBL producer strain out of 100 randomly selected strains.

Occurrence and Enumeration

The comparative total viable counts (log cfu/ml) of *V. cholerae* in raw, treated and tap water samples are presented in table: 1. The Mean values of S1 and S2 readings are presented in graphical form (Fig.1).The results revealed that 100% occurrence of *V. cholerae* was observed in all the raw water, treated water and tap water samples, throughout the year.

All the 24 raw water samples harbored high densities of *V.cholerae* throughout the year, which ranged from 2.0 log cfu/ml (Feb, S1) to 2.6 log cfu/ml (July, S1). Seasonal studies of the counts (Fig.2) showed that the highest count (2.5 log cfu/ml) was recorded during rainy (July to Oct 2013) season, attributable to influx through runoff of microorganisms originating from vegetation decay, municipal sewage, garbage, domestic and fecal waste into the Jaju Sagar Dam making it highly contaminated .The dense growth may also be due to the dual effect of required salinity and optimum temperature of raw water. Whereas least count (2.2 log cfu/ml) was recorded during winter (Nov-Feb 2013), probably due to the lower temperature of water which might decelerated the growth rate of the organisms. During summer months (Mar-June 2013) the average count was 2.3 log cfu/ml, higher than colder months, this clearly indicates that the high temperature of water might enhanced the growth and reproduction of the organisms. Our results slightly vary with that of Haque et al. [22] who reported highest counts in summer season followed by monsoon and winter. Our results are in agreement to many of the previous studies. Rashid et al. [38] also detected *V. cholerae* in drinking water reservoirs of Azerbaijan and reported its high incidence during summer months. His calculations on environmental parameters suggested that higher temperature of warmer months is responsible for the increased detection of *V. cholerae*. Nair et al. [37] studied ecology and seasonal incidence of *V. cholerae* in a man-made freshwater lake of Calcutta and concluded that temperature and, to a certain extent, pH is the important factors which affect the densities of *V. cholerae*. The data of present study demonstrate a clear trend confirmed by other investigators [32, 24, 15, 46]. Therefore, it is inferred that the rainy and summer months may have the closest optimum temperature range, favorable for the growth of *V. cholerae*. Thomson et al. [45] studied prevalence of *Vibrio* spp. in drinking water and environmental samples in Vellore, South India and found that 41 % of drinking water samples contained Non-01, Non-0139 strains of *V. cholerae.* Shittu et al. [42] also reported high numbers of *V. cholerae* in drinking water sources. Hatha et al. [23] also studied high prevalence of *V. cholerae* in Kumarakom lake. From all these findings it may be inferred that *V. cholerae* is the normal inhabitant of drinking water sources and other fresh water aquatic bodies.

Table 1: Log CFU/ml counts of *V. cholerae* for the year 2013

Months	Raw water		Treated water		Tap water	
	S1	S2	S1	S2	S1	S2
January	2.2	2.2	1.4	1.3	1.9	2.0
February	2.0	2.0	1.0	1.2	1.8	1.9
March	2.3	2.3	1.6	1.6	2.0	2.1
April	2.2	2.1	0.5	1.4	2.0	2.1
May	2.4	2.4	1.6	1.7	2.1	2.2
June	2.3	2.3	1.6	1.6	2.1	2.1
July	2.6	2.6	1.7	1.8	2.2	2.3
August	2.5	2.5	1.5	1.6	2.2	2.2
September	2.5	2.5	1.5	1.0	2.0	1.9
October	2.3	2.3	1.6	1.6	1.9	2.1
November	2.3	2.2	1.3	1.6	2.1	2.2
December	2.3	2.2	1.6	1.4	2.1	2.0

S1- First sample of the Month, **S2**- Second sample of the Month after 15 days.

Each value is a log number calculated from the **MEAN** of CFU counts of triplicates

The total viable counts in treated water samples ranged from 0.5 log cfu/ml (April, S1) to 1.8 log cfu/ml (July, S2).Comparative analysis showed that all the treated water samples showed decreased amounts of counts, as compared to that of raw water samples, but the counts were above the guideline value of zero *V. cholerae*, as prescribed by the WHO (2011) and BIS (2012). This reveals the inefficiency of the filtration and chlorination steps at the Hingoria Treatment Plant to produce the water of an acceptable quality. All the tap water samples were found to be unsatisfactory for drinking because according to recommendations from the WHO [51] and BIS [8], and previous studies, drinking water must be free from any pathogen. The log cfu/ml counts ranged from 1.8 (Feb, S1) to 2.3 (July, S2).The tap water samples showed higher counts than the treated water samples. These increased counts may be either due to the fact that the amount of chlorine is not adequate to avoid the chances of post contamination or to the infiltration of contaminated water through cross-connection, leakage points and back siphonage. In piped supplies, discontinuity increases the likelihood of contamination as the risk of back siphonage into the distribution network is increased when pipes are at lower pressure than the surrounding soil, which often contains leaked out effluent from leaking sewers.

Morphological, Cultural & Physiological studies-

Gram staining of isolates showed short, straight and comma-shaped gram negative rods, singly and in short chains. Appearance of cloudiness and thin spreading filaments in mannitol motility test medium confirmed the motility of the cells. Overnight growth (18 to 24 hours) of *V. cholerae* on TCBS produced large (2 to 4mm in diameter), slightly flattened, yellow colonies with opaque centers and translucent peripheries. The yellow color was due to the fermentation of sucrose in the medium. On prolonged incubation, the yellow colonies became green. On nutrient agar plates the colonies were white, opaque, round with entire margin and convex elevation. Isolates showed filiform type of growth on nutrient agar slant and in nutrient broth it produced pellicle type of surface growth, granular type of sub-surface and flaky type of sediment growth. Results of physiological studies are presented in table: 2.

Table 2: Biochemical characteristics of *V. cholerae* isolates and typestrain

Characteristics	*Vibrio cholerae*	
	Typestrain	**Isolated strains**
Motility	+	+
Growth in 0% NaCl	+	+
Growth in 1% NaCl	+	+
TCBS	+	+
O/F test	Fermentative	Fermentative
Catalase	+	+
Oxidase	+	+
Arginine	-	-
Lysine	+	+
Ornithine	+	+
Urease	-	-
Amylase	+	+
Gelatinase	+	+
Nitrate reduction	+	+
Indole	+	V
MR		V
VP	+	V
Simmon Citrate	+	+
Mannitol	+	+
TSI agar-		
Glucose	+	+
Gas from glucose	-	-
Sucrose	+	+
Lactose	+	V
H_2S	-	-

+ = 90 to 100% of the isolates were positive;

- = 0 to 10% of the isolates were positive; **V** = variable reaction

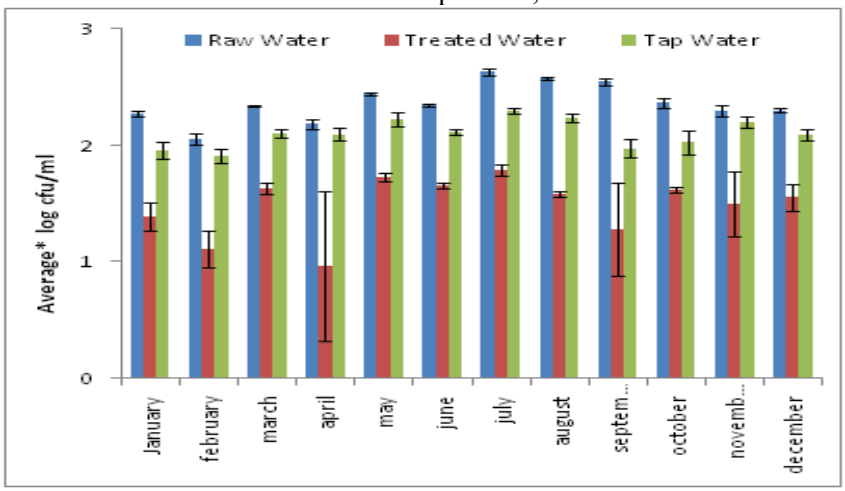

Fig 1: Average Log cfu/ml (*Mean of S1 &S2) of *V. cholerae* in Raw, Treated and Tap water.

*Mean of S1 &S2, The bars show Standard Deviations

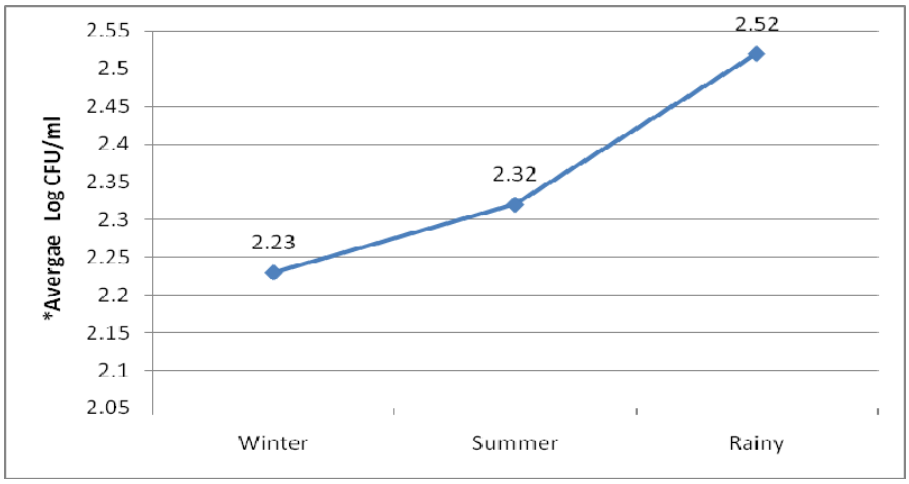

Fig 2: Seasonal shifts in *V.cholerae* counts in raw water samples
***Mean of four readings**

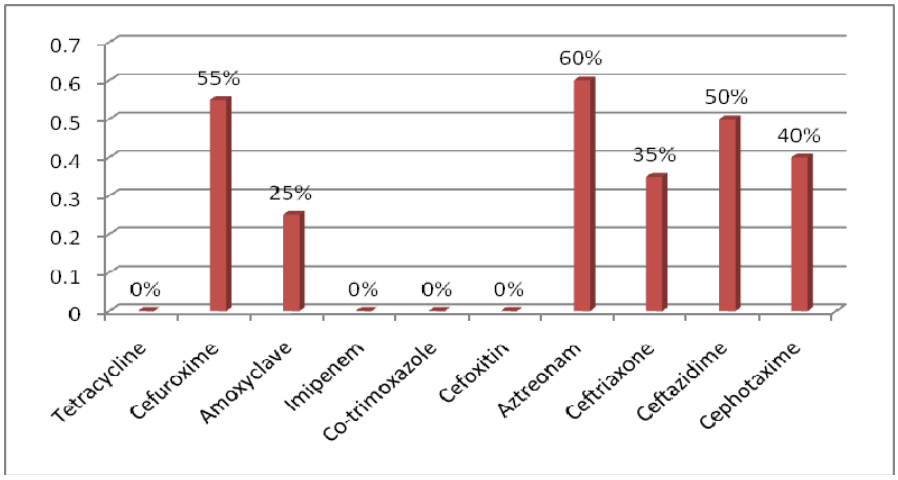

Fig 3: Antibiogram (resistance percentages) of *V. cholerae* isolates tested against various antibiotics

Antibiotic susceptibility pattern

The study of antibiogram (Fig.3) on 100 isolates revealed that all the tested strains (100%) were sensitive to tetracycline .Our results are not in agreement to many of the previous studies. Contrasting results were obtained by Islam et al. [26] who reviewed the tetracycline resistance pattern of *V. cholerae* isolated from diarrheal patients in Dhaka, Bangladesh during 2000-2009 and observed rapid increase of resistance to tetracycline from 2004 to 2009. Mhalu et al. [36] also noticed rapid emergence of tetracycline resistance in *V. cholerae* isolates from patients du e to extensive use of the drugs. There are also other reports of tetracycline resistant *V. cholerae* strains responsible for major epidemics of cholera in Latin America, Tanzania, Bangladesh and Zaire [44,47] found a significant increase in the proportion of tetracycline resistant isolates when he compared the antimicrobial susceptibility patterns of the *V. cholerae* 01 isolates obtained in 1997 with those in 1999. Our result is consistent with that of Dalsgaard et al. [16]**.** Low level of tetracycline resistance (4.34-15.38%) was also obtained in a study of Chandigarh by Chander et al. [10] likewise most *V. cholerae* isolates were sensitive to tetracycline in a study by Kaur et al. [28]. In a recent study of Materu et al. [34] from eastern African countries between 1994 and 1996, about 80% to 90% of the isolates in Kenya and southern Sudan, and 65 to 90% of isolates in Somalia were sensitive to tetracycline .However, during the same period, 100% of isolates from Tanzania and Rwanda were resistant to tetracycline [34]. Our findings also corroborated with other studies [41, 43, 27], which also reported isolates being sensitive to tetracycline. No strain was found resistant to co-trimoxazole, the same result was also reported by Chander et al. [10] who observed a decrease in co-trimoxazole resistance from 100 to 83 % over the years 2003, 2004 and to 0 per cent resistance in 2005. While study of Garg et al. [21] from Calcutta reported an increase in resistance to co-trimoxazole from the year 1994 onwards. Likewise, 100 % co-trimoxazole resistant was also seen in the study of Islam et al. [25].

All the strains were sensitive to imipenem, 100 % imipenem sensitivity was also observed in a study of Ismail et al. [25] in Mpumalanga, South Africa. 55% strains were resistant to cefuroxime, 25% to amoxyclav. 95% strains were sensitive and 5% was intermediate to cefoxitin. Standard CLSI breakpoints for erythromycin do not exist.60% of the *V. cholerae* isolates were resistant to aztreonam, 50% to ceftazidime, 40% to cephotaxime, 35% to ceftriaxone. The strains (n=60) resistant to third generation cephalosporins were selected for further detection of the ESBL by double disc synergy test (DDST). But in contrast to many previous studies on ESBL detection of *V. cholerae* strains that reported a high incidence of ESBL, all the 60 tested strains gave negative double disc synergy test (DDST) hence were non-ESBL producer. Non –ESBL third generation resistant *V. cholerae* was also reported by Mandal et al. [33] from a child patient of cholera but the strain was positive for the Amp C – disc test ,modified Hodge test, and EDTA disk synergy test.

CONCLUSION

We draw following peculiar conclusions from the current study-

1. Our study is the first report of the occurrence of *V. cholerae* in Jaju Sagar Dam. This shows that the dam is the potential reservoir of *Vibrio cholerae*, but whether the strains are cholera causing (serogroup O1 and O139) or not, is a question of further research because we have not gone through the serotyping and toxicity testing of the isolated strains. In a condition of the strains being non-cholera causing, then also the inhabitants of Neemuch may be in great risk because *Vibrio cholerae* non- toxigenic non-O1/non-O139 strains may also cause small outbreaks and sporadic cases of cholera, as well as extra intestinal infections [11, 30, 39, 31, 40]. These mild cases often go undiagnosed.
2. The presence and percentage of *V. cholerae* in treated and tap water revealed inefficient working of Hingoria treatment plant in removal of pathogenic bacteria from the raw water. It is highly recommended that Hingoria Treatment Plant must be upgraded and subjected to technical assessment by a competent lab technician for the regular monitoring of the microbiological quality of the treated water before supplying to the community, before an outbreak of epidemic occurs.
3. No ESBL positive *Vibrio cholerae* was detected during the DDST assay profiling of 60 strains that were resistant to third generation cephalosporins. Due to the lack of a genetic analysis of these strains, the exact nature of the underlying mechanisms mediating the resistance is not clear.

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