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Simple method devised for rapid isolation and identification of *Vibrio cholerae* from water resources of Sunsari District, Nepal

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Abstract
Cholera is a gastrointestinal disease caused by pathogenic strain of *Vibrio cholerae*, the disease clinically manifested by rice-water diarrhea, nausea and vomiting. This study aimed to study the incidence of Vibrio species and employ simple method for rapid detection of *Vibrio cholera*e from water samples of Sunsari, Nepal. Identification of *V. cholera*e through biochemical tests requires extensive labor and costs. In resource limited laboratories, isolation and identification of *V. cholera*e often becomes difficult. Therefore, this study also aimed for selecting scope of this methodology as a scientific outcome for rapid isolation and identification of *Vibrio cholerae*. A total of 100 water samples were collected from Sunsari district in which 25 samples were collected from sewage, 25 from pond, 25 from tap and 25 from tube well. The samples of collected water were sent to the microbiology laboratory of Central Campus of Technology maintained in ice cold box and were enriched in Alkaline Peptone water and selectively isolated from TCBS agar and NA agar without NaCl. Pathogens were isolated and identified by conventional microbiological techniques. Out of 100 water samples collected, sucrose fermenting *Vibrio* species were isolated only from 16 water samples. Further the selective isolation of *V. cholerae* from nutrient agar without NaCl isolated 6 isolates from sewage samples and 3 isolates from pond samples. The distribution of *Vibrio cholera* in the water sample was found to be 9%, distribution of *V. alginolyticus* was found to be 4% and distribution of *V. fluvialis* was found to be 3%. In this study, non-sucrose fermenting Vibrio species were not isolated from the water samples. However, sucrose fermenting Vibrio species was obtained with yellow pigmentation in TCBS agar medium. The yellow pigmented colonies of Vibrio isolates recovered from TCBS and even from Nutrient Agar devoid of sodium chloride provided sufficient evidence of *V. cholerae* after series of other biochemical tests. This study concludes that yellow colonies (sucrose-fermenting) of Vibrio from TCBS agar medium that can grow on nutrient agar without added NaCl and which exhibit a positive oxidase reaction can be confidently identified as presumptive *V. cholerae*. In resource-constrained environments, this simple method can reduce the labor cost, chemicals and time-consuming procedure of performing multiple biochemical and molecular assays for identification.

Keywords: Cholera, lab diagnosis, Sunsari, TCBS, *Vibrio cholerae*, Water

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Introduction
Vibrio is a gram-negative rod-shaped bacterium that is an inhabitant of water sources. The species distribution of this bacterium is dependent on salt concentration and temperature of water [1]. Among several species, *V. cholerae* , *V. parahaemolyticus* and *V. vulnificus* are known to be pathogenic. Strains of V. cholerae of serogroups O1 and O139 are known to be associated with epidemic potential whereas non-O1 and non-O139 strains are identified with mild diarrheal cases [2]. Cholerae symptoms include abrupt onset of watery diarrhea (rice watery stool), nausea, vomiting, and intestinal disorders with abdominal pain [3]. Cholera is clinically manifested by rapid loss of water that accounts for dehydration, polydipsia, hollow tear troughs, decreased blood pressure, weak pulse, renal failure, seizures, coma, and death [4].

V. cholerae are known to be associated with several epidemics and pandemics. In underdeveloped nations, the fecal contamination of food-water and poor sanitary habits are associated with cholera epidemic [5]. Therefore, the aim of this study was to study the incidence of Vibrio species and devise simple method for rapid detection of Vibrio cholera from water samples of Sunsari, Nepal.

Methods and materials
Study site
The study area was Sunsari District (Latitude 26° 37' 39.19" N and longitude: 87° 10' 55.82" E) of Province No.1, Nepal. The district is situated in Terai region and covers an area of 1,257 km². Sunsari district is situated at an altitude up to 6600 ft. from sea level.

Study design
This study was designed to setup from November 2018 to January 2019. In this study water samples were collected from different regions of Sunsari district, Nepal. During the study period a total of 100 water

samples were collected from four available sites (Pond, sewage, tap and tube well) of districts rich in water resources. In this study, same frequency of samples was collected from four different places in which 25 water samples were collected from sewage, 25 from pond, 25 from tap and 25 from tube well from random areas of Sunsari district to have uniform distribution of different samples. Sites of water samples were selected by simple random sampling and lottery method. About 100 mL water was collected in a sterile BOD sample bottle and was transported to microbiology laboratory of Central Campus of Technology, Dharan on the same day maintained in ice cold chain and processed within 3 hours of collection.

Societal information

The socio-demographic activities of people affecting water sanitation were observed and information were collected from local residents through a semi- structured Questionnaire.

Inclusive and exclusive criteria

Water samples from rivers, ponds, tap and sewage were included in this study.

Screening *V. cholerae* from water

Isolation of *V. cholerae* from water was performed as described by CDC (1994) [6]. Membrane filters technique was most appropriate for clear sample that did not contain debris, mud or silt. Clean, non-cloudy and sediment free water from tap and tube well was filtered through membrane filtration technique. In this method, 100 mL of water sample was passed through 0.22 µm membrane filter (HiMedia, India) attached to suction flask. With the help of a sterile forceps, the Membrane filter was inoculated in test tube containing 10mL of sterile alkaline peptone water of pH-8.6 (APW) (HiMedia, India) and vortexed for 30 sec. The enrichment of bacteria from pond and sewage was performed by inoculating 1 mL of sample water in 9 mL alkaline peptone water.

The enriched APW culture tubes were incubated at 37°C for 6 hours and then the topmost layer of enriched aliquot was inoculated on thiosulfate citrate bile salts sucrose (TCBS) agar media (HiMedia, India) and streaking over the TCBS agar surface was done with the help of sterile inoculating loop. The inoculated culture media were incubated at 37°C for 18-24 hours. After overnight incubation, the Vibrio like yellow colony, 2 to 4 mm in diameter, slightly flattened with opaque centers were chosen and cultured on nutrient agar without NaCl and incubated at 37°C for 16-24 hours. In routine microbiology tests, the bacterial colonies obtained from TCBS are cultured in Nutrient Agar with NaCl alongside overnight incubation and further the isolated colonies are biochemically tested which requires lengthy cost, reagents, time and labor. Most of the biochemical tests require overnight or 18-24 hours long incubation period. In this regard, suspected isolated *Vibrio* culture from TCBS directly grown in NA devoid of NaCl provides presumptive identification of *V. cholerae* without further biochemical tests.

Biochemical test for identification of *V. cholerae*

After incubation, the nutrient agar (NA) plate with the growth of colorless, glistening, translucent colonies provided presumptive identification of *V. cholerae* . For further confirmation, Gram staining and biochemical tests like oxidase test, string test, MR test, citrate test, indole test, esculine hydrolysis and arginine dihydrolase activity were performed as described by CDC (1994) [6]. For Gram staining and biochemical tests, the culture was taken from nutrient agar without NaCl (non-selective media). Sucrose fermenting colonies (yellow pigmented) from TCBS which were not recovered in NA devoid of NaCl were further cultured in Nutrient agar with 0.5% NaCl and subjected for biochemical tests.

Quality Control

V. cholerae O1 El Tor strain N16961 was used in this study as positive control. Reagents and culture media were regularly monitored for their performance. Equipment was standardized, optimized and its performance was checked through pretesting of positive and negative controls.

Data analysis

The data was documented in MS-EXCEL 2010 and was analyzed using SPSS version 16.0 software. Statistical significance was established at $P < 0.05$ with 95% confidence interval.

Results

Out of 100 water samples collected, sucrose fermenting *Vibrio* species were isolated only from 16 water samples (9 isolates from raw sewage, 6 isolates from pond devoid of any sewage connections and 1 isolate from public tap water). None of the non-sucrose fermenting *Vibrio* species were isolated from TCBS. Further, the selective isolation of *V. cholerae* from nutrient agar without NaCl isolated 6 isolates from sewage samples and 3 isolates from pond samples. The incidence of

Table 1. *Vibrio* species from culture media.

Sources (Total sample)	Sucrose fermenters	Isolates from NA without NaCl (*V. cholerae*)	Isolates from NA with NaCl (*V. alginolyticus*)	Isolates from NA with NaCl (*V. fluvialis*)	P-value
1. Sewage (S) (25)	9	6	2	1	
2. Pond (P) (25)	6	3	1	2	P=0.0
3. Tap water (25)	1	0	1	0	01
4. Tube well (25)	0	0	0	0	
Total	16	9	4	3	

Vibrio cholera in the study sample was reported to be 9%, *V. alginolyticus* to be 4% and *V. fluvialis* to be 3%. However, in this study *V. cholerae* was not isolated from tap and tube well water **(Table 1)**. The biochemical tests: oxidase test, string test, citrate test, indole test, MR test, esculine hydrolysis, arginine dihydrolase test confirmed the isolates recovered from NA without NaCl to be *V. cholerae* (**Table 2**). The biochemical tests of sucrose fermenting 7 *Vibrio* isolates from TCBS which did not grow in nutrient agar devoid of NaCl reported 4 isolates to be *V. alginolyticus* and remaining 3 isolates to be *V. fluvialis*. There was significant difference in distribution of *Vibrio cholera* from different water samples (P=0.001).

Table 2. Biochemical tests of isolated *V. cholerae* .

Biochemical Test	Bacterial isolates									
	S1	S2	S3	S4	S5	S6	P1	P2	P3	C
Oxidase Test	+	+	+	+	+	+	+	+	+	+
String Test	+	+	+	+	+	+	+	+	+	+
Citrate Test	+	+	+	+	+	+	+	+	+	+
Indole Test	+	+	+	+	+	+	+	+	+	+
MR	-	-	-	-	-	-	-	-	-	-
Esculine hydrolysis	-	-	-	-	-	-	-	-	-	-
Arginine dihydrolase	-	-	-	-	-	-	-	-	-	-

Note: Isolates S1, S2, S3, S4, S5, S6 were isolated from sewage whereas isolates P1, P2 and P3 were isolated from Pond. C is the quality control strain; *V. cholerae* O1 El Tor N16961 strain used in the study

Table 3. Characteristics pertaining to water contamination at study sites.

Characteristics	Pond	Tap water	Tube well water
1.Open human Defecation	Yes	No	No
2.Animal washing and defecation	Yes	No	No
3. Wastes dumping sites	Yes	No	No
4. Source of drinking water	Yes	Yes	Yes
5. Use of sample site for religious practices	Yes	No	No
6. Water treatment before drinking	No	No	No

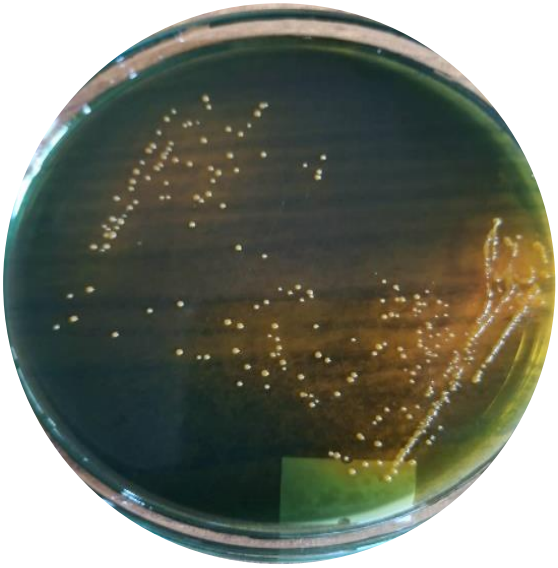

Figure 1. *Vibrio* sp. on TCBS media with yellow colony, 2-4mm in diameter, slightly flattened with opaque centers.

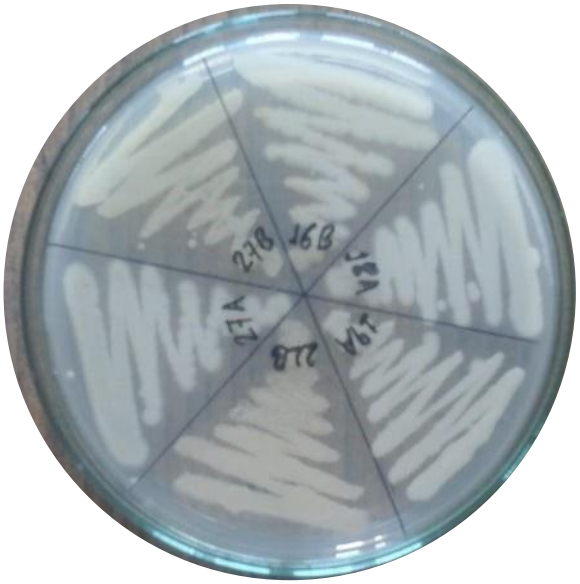

Figure 2. *V. cholerae* with colorless, glistening, translucent colonies on Nutrient Agar without NaCl.

Discussion

Cholera has been a disease of great epidemic potential for the last many centuries and now has become endemic in many countries [7]. Cholera outbreaks have been reported frequently from different parts of Nepal [8]. *V. cholerae* 01 strain associated with epidemic outbreak of Cholera has been known to establish in aquatic environment posing threat to public health [9].

Multidrug Resistance *V. cholerae* EL Tor possessing cholera toxin (ctx) identified with diarrheal illness from three districts from Nepal was similar with strains related with cholera outbreak in Bangladesh and Haiti [10].

V. cholerae and *V. mimicus* can only grow in media that lacks sodium chloride while other species of *Vibrio* are strictly halophilic in nature [11]. *V. cholerae* can ferment sucrose in TCBS medium producing yellow colony whereas *Vibrio mimicus* fails to ferment sucrose and hence produce green pigmented colony on TCBS [12]. All the colonies growing on TCBS media with yellow colony are not necessarily *V. cholerae*. The ability of *V. cholerae* to grow on culture media devoid of sodium chloride helps in selective isolation and identification of *V. cholerae* from other Vibrio species [13].

In this study the presumptive isolation and identification of *V. cholerae* was made from media devoid of NaCl within two days of sample collection and processing. This study was consistent with the study that has demonstrated that *V. cholerae* can be selectively isolated from media devoid of sodium chloride [14]. The main principle of this study was the enrichment of *Vibrio* in alkaline peptone water and its selective isolation from TCBS and NA without NaCl. The biochemical tests like esculine hydrolysis and arginine dihydrolase are efficient techniques for identifying *Vibrio cholera* from aquatic samples [15]. In this study, even arginine dihydrolase test stood key tool for differentiating *V. alginolyticus* (Arginine dihydrolase-negative) from *V. fluvialis* (Arginine dihydrolase-positive). The Gram staining, string test and oxidase test along with serogrouping, biotyping and serotyping can be performed minimum from second day of sample processing. However, PCR based methods can provide sensitive result much rapidly within 6-12 hours but conventional methods are only the option in resource limited areas. However, antimicrobial susceptibility test can only be accessed through conventional culture method which is most important in clinical settings. Serogrouping, biotyping and serotyping of *V. cholerae* need to be performed whenever there are reported and outbreak cases which provides evidence for clinical and epidemiological studies.

V. cholerae differs from other Vibrio species in that it can grow in nutritional broth without the addition of sodium chloride [16]. In this study the identification of *V. cholerae* from other Vibrio species was based on three major trait features like sucrose fermentation, non-requirement of additional Na^+ for growth, and presence of oxidase which was in consistent to the findings of other similar study [17]. Most sucrose-positive halotolerant or halophilic Vibrio were eradicated when grown on nutritional agar without additional NaCl. It is also concluded that isolation of *V. cholerae* from media devoid of NaCl is simple and rapid method of isolation and identification which was in agreement to the present study [14]. However, some limiting factors may exists, study reports that some *V. cholerae* strains, have been reported of not being able to ferment sucrose. These strains would not be detected in conventional studies as only sucrose fermenting Vibrio from TCBS are considered for further identification of *V. cholerae* [18]. Some *V. cholerae* strains which are strictly halophiles might also remain in shadow during isolation through media devoid of NaCl. In addition, routine conventional microbiological techniques are not capable of isolating Viable but non-culturable (VBNC) state of *V. cholerae* [19]. In this regard, only the resource rich settings with the polymerase chain reaction provides high specificity and accuracy for identifying Vibrio species and can even distinguish its biotypes.

According to the study, the prevalence rate of *V. cholerae* from water samples of Bhaktapur was 5% [20] and from Kathmandu, the prevalence was 11.11% [21] which were in agreement to the prevalence of the current study. However, the prevalence of *V. cholerae* was 0.84% in one study from drinking water of Kathmandu [22]. The filthy pond with algae growth might be an organism's reservoir [23]. In Nepal, the period between mid-june to mid-july had the highest incidence of Cholera [24]. In 2012 A.D., diarrhea outbreaks in three districts of Nepal were due to transmission of multidrug resistant *V. cholerae* El Tor possessing cholera toxin (ctx) B-7 allele, which is clonal and related closely with *V. cholerae* associated with cholera in Bangladesh and Haiti [25]. The increased prevalence and isolation rates of *V. cholerae* in Nepalgunj (Banke), Dang, and Dhangadhi (Kailali) might be attributed to contaminated drinking water, poor management of water pipes, sewage, and excreta, and a less sanitary environment [26].

In one study *V. cholerae* was found in two of the patients from Sunsari. In the same study, eight of the thirteen water samples were determined to be unfit for human consumption. Contamination of the source of drinking water and the major reasons of such epidemics were unsanitary behaviors. It is, thus, necessary that gastro enteritis outbreaks may be avoided simply by encouraging cleanliness and sanitary practices behaviors involving drinking water and defecation [27]. The presence of heterotrophs and the coliform in the drinking water marketed in eastern Nepal is a serious

concern for public health [28]. *V. cholerae* isolated from pond and sewage water samples of sunsari district could pose a severe health hazard to humans who either come in contact with or consume water from the contaminated site. In addition, the lack of safe drinking water, awareness and poor sanitary practices among people of rural areas of sunsari makes them more prone to the infections. Water borne diseases among underprivilege family and community remains in shadow until it leads to community burden leading heavy mortality and morbidity. Microbiological water surveillance in these rural settings have never gained importance and attention from the concerned authority that alarms the possibility of future outbreak of disease.

In this study the possible cause behind the water contamination was also studied. The necessary information was collected from the local residents through Questionnaire. It was found that the local villagers of Terai observe chhath festival (local ethnic festival that includes Fasting, Prayers and religious rituals by taking bath in ponds/ rivers water). In this course of ritual, the activity of open defecation results in fecal contamination of water. In this report, the pond of this study sites was even used for many ritual performance, open defecation, animal washing and even for drinking purpose. Human activities like disposing human and animal wastes have been identified as the main cause behind deteriorating water resource that leads pond water to be a potent source for cholera transmission [8]. Outbreak of Cholera in Saptari VDC of Nepal was associated with use of pond water which was contaminated by *V. cholerae* [23]. One study reported that diarrheal outbreak in Rautahat District of Nepal was associated with *V. cholerae* O1 Ogawa serotype, which was result of fecal contamination of drinking water from nearby sewage [29]. Hence, these finding addresses the cause and route behind fecal contamination of water which can lead to cholera outbreak. Therefore, Studies suggest promotion in safe drinking water is fundamental approach to prevent morbidity and mortality induced by cholera in near future [30]. Maintenance of safe water chain a key approach for preventing cholera outbreak in near future.

Conclusions

This study concludes that yellow colonies (sucrose-fermenting) of Vibrio from TCBS agar medium that can grow on nutrient agar without added NaCl and which exhibit a positive oxidase reaction can be confidently identified as presumptive *V. cholerae*. This method can reduce the labor, reagents, cost and time-consuming process of conducting several biochemical and molecular tests in resource limited settings.

Abbreviations

APW: Alkaline Peptone Water
BOD: Biological Oxygen Demand
CDC: Centre for Disease Control and Prevention
NA: Nutrient Agar
NaCl: Sodium Chloride
PCR: Polymerase Chain Reaction
TCBS: Thiosulfate Citrate Bile Salts Sucrose
VDC: Village Development Committee

Limitations

Molecular methods were not performed for identification of bacteria. Serotyping, Biotyping and Antibiotics susceptibility test of isolated pathogens were not done.

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Author's Contribution:

BKS participated in study design, sample collection, sample processing, organism identification, data analysis, intellectual content design and result interpretation. JS participated in sample collection, sample processing, and proof-reading manuscript for intellectual content. Both the authors drafted the manuscript and agreed for its publication.

Conflict of interest

Author declares no conflict of study.

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