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RESEARCH ARTICLE
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ENVIRONMENTAL GROWTH FACTORS AND IDENTIFICATION OF V. CHOLERAE FROM DIARRHEAL PATIENTS IN BALOCHISTAN

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ABSTRACT

Background: Cholera remains a significant public health concern in developing countries such as Pakistan, caused by Vibrio cholerae, a Gram-negative, motile, curved rod-shaped bacterium that thrives in aquatic environments. Although over 200 serogroups have been identified, only O1 and O139 are responsible for epidemic outbreaks.

Objective: This study aimed to evaluate the identification of V. cholerae isolates from diarrheal patients using biochemical and serological methods, alongside assessing the influence of environmental factors, particularly temperature and pH, on their growth.

Methods: from September 2022 to September 2023, 888 stool samples were collected from suspected cholera patients across hospitals in Balochistan. The samples were cultured on thiosulfate-citrate-bile salts-sucrose agar, and colonies suggestive of V. cholerae were further examined by Gram staining, biochemical tests, and serological identification. Additionally, the growth pattern of isolates was tested at varying temperatures (10°C, 15°C, 25°C, 37°C, and above 50°C) and pH levels (4–12) to determine optimal environmental conditions.

Results: Of the total samples, 66 tested positive for V. cholerae. Serotyping confirmed that 55 isolates belonged to serogroup O1 Ogawa, while 11 (8.3%) were identified as non-O1/non-O139 serogroups, showing no agglutination with Inaba, Ogawa, or O139 antisera. Temperature greatly affected bacterial growth, with optimal proliferation at 37°C, moderate growth at 25°C, and no growth at 10°C, 15°C and 50°C. The organism demonstrated maximum growth in an alkaline environment, particularly between pH 8 and 12, with negligible growth at pH 7, and complete inhibition at pH 5.

Conclusion: The findings confirm that V. cholerae multiplies efficiently in aquatic environments under moderate temperatures and alkaline conditions, facilitating its survival and transmission. Routine laboratory diagnosis employing both culture-based biochemical tests and serological identification is crucial for detecting circulating V. cholerae strains and implementing effective public health interventions in Balochistan.

Introduction

Cholera is a life-threatening intestinal infection marked by copious watery diarrhea and vomiting, leading rapidly to dehydration, metabolic acidosis, and circulatory failure if untreated. The disease results from consuming food or water contaminated with Vibrio cholerae, a motile, curved, gram-negative rod-shaped bacterium that tests positive for oxidase and catalase enzymes and ferments glucose without gas production. **(Harris et al 2005)** V. cholerae is naturally present in marine and estuarine waters globally, where it can persist and multiply in contaminated water sources. Additionally, asymptomatic human carriers act as reservoirs, particularly in endemic areas, silently propagating the infection **(Igbinosa and Okoh, 2008).**

The organism grows best in alkaline conditions, with an optimal pH between 8.0 and 10.5, although it can tolerate up to pH 11 but is destroyed by stomach acid. While most bacteria prefer neutral pH, fluctuations in pH greatly influence bacterial enzymes and cellular processes, with extreme pH levels hindering microbial proliferation, as shown in multiple studies**.(Russell et al., 2003)**

Temperature profoundly affects bacterial viability by altering membrane integrity, enzyme function, ribosomal activity, and protein stability. Vibrio species, including V. cholerae, grow within a temperature range of 20°C to 40°C. Their occurrence in aquatic habitats depends on salinity and nutrient availability, explaining their selective prevalence in estuarine and coastal environments. Therefore, despite sufficient nutrients, environmental factors such as temperature, pH, and osmotic pressure remain crucial for bacterial survival and growth. **(Holmgren et al., 1973; Kaper et al., 1979).**

Laboratory confirmation of cholera typically involves culturing stool or rectal swab specimens on selective media to isolate and identify the pathogen. Commonly used media include thiosulfate citrate bile salts sucrose (TCBS) agar, tellurite taurocholate gelatin agar (TTGA), and CHROMagar, with TCBS being the standard medium worldwide for isolating V. cholerae. Enrichment in alkaline peptone water is often employed to enhance isolation. Confirmed colonies undergo biochemical testing or subculture onto non-selective media for further characterization**.(McCormack WM, et al 1974)**

Biochemically, V. cholerae differs from Enterobacteriaceae by yielding a positive oxidase reaction, turning purple due to cytochrome oxidase acting on tetramethyl-p-phenylenediamine dihydrochloride. It ferments sugars such as glucose, sucrose, maltose, mannitol, and trehalose, with variable gas production. The bacterium decarboxylates ornithine and lysine but not arginine. Classical strains test negative in the Voges-Proskauer test, which detects diacetyl production from acetylmethylcarbinol in the presence of α-naphthol and KOH, producing a pink-red color. Additionally, V. cholerae often forms a characteristic mucoid string in 0.5% sodium deoxycholate, a useful diagnostic feature. **(Weil et al., 2015).**

This study is essential for Balochistan because cholera remains an underreported yet recurrent cause of diarrheal outbreaks in the province due to inadequate water sanitation, poor hygiene infrastructure, and limited diagnostic facilities. Understanding the growth requirements, environmental persistence, and biochemical identification of V. cholerae will aid local health authorities in prompt diagnosis, outbreak control, and implementation of targeted public health interventions. Such knowledge can significantly reduce morbidity and mortality, particularly among vulnerable populations including children and rural communities who rely on untreated water sources.

Materials and Methods

A total of 888 stool specimens were obtained from patients presenting with untreated severe diarrhea or showing clinical signs of significant dehydration. These samples were collected from different hospitals across Balochistan during the period from September 2022 to September 2023.All samples were transported in Cary-Blair transport medium (Oxoid, Hampshire, UK) to the Microbiology laboratory Bolan medical Complex Hospital Quetta, for further processing.

Samples were enriched in alkaline peptone water for 6 hours and then streaked on thiosulfate citrate bile salts sucrose (TCBS) agar (Oxoid Ltd, Hampshire, UK). The plates were incubated at 37℃ for 24 hours **(Winn et al., (2005).** Typical golden-yellow colonies were subjected to Gram's staining and identified by biochemical and serological methods.

Biochemical and carbohydrate fermentation tests were carried out to characterize the isolates. These included oxidase, catalase, indole, Voges–Proskauer, methyl red, and citrate utilization tests. Fermentation ability was assessed using glucose, mannitol, sucrose, sorbitol, trehalose, maltose, inositol, and lactose. In addition, biotyping of the isolates was performed by determining resistance to polymyxin B and examining hemolysis of sheep erythrocytes, which

Serological identification was performed by slide agglutination assay using polyvalent antisera (Murex Diagnostic Ltd., UK) for serogroup O1 (Ogawa and Inaba) and serogroup O139 (Dienka Sieken Co. Ltd., Japan). Monovalent antisera were used for precise serotype identification.
To evaluate the influence of temperature on the growth of Vibrio cholerae, the confirmed isolates were incubated for 24 hours at a range of temperatures (10℃, 15℃, 25℃, 37℃, and 50℃). After incubation, bacterial growth was carefully monitored and recorded. This approach helped determine the optimal temperature conditions that support multiplication as well as the threshold limits where growth was inhibited.

The influence of pH on Vibrio cholerae growth was examined by preparing culture media adjusted to different pH levels (5, 7, 8, 11, and 12). The required pH was obtained using 4% NaOH and 1M HCl, after which the media were sterilized by autoclaving. Following inoculation, the plates were incubated at 37℃ for 24 hours, and colony development was subsequently evaluated on TCBS agar to determine the effect of varying pH conditions.

Results
Out of the total samples examined, 66 isolates were identified as *Vibrio cholerae* based on their morphological characteristics. On TCBS agar, the colonies appeared flat, smooth, yellow, and measured approximately 2–3 mm in diameter, while microscopic examination showed Gram-negative, curved rod-shaped bacteria with a characteristic comma-like appearance.
Biochemical characterization of the isolates revealed consistent patterns. All isolates tested positive for oxidase, catalase, indole, methyl red, and citrate utilization, while none showed urease activity. Carbohydrate fermentation tests further confirmed their identity, as the strains were able to ferment glucose, mannitol, sucrose, and trehalose. However, no fermentation was observed with lactose, sorbitol, or inositol. These results supported the typical biochemical profile of Vibrio cholerae, strengthening the confirmation of the isolates shows in Table 1.

In biotyping identification, no inhibitory zones were observed around the polymyxin discs, indicating the presence of El Tor strains.

Serological analysis revealed that the serogroups of V. cholerae isolates were identified through agglutination with polyvalent antisera, followed by monovalent antisera to determine serotype. Among the 66 confirmed isolates, 55 belonged to serogroup O1, while 11 were non-O1/non-O139. None of the isolates agglutinated with O139 antisera shows in Fig. 1.
Growth at different temperatures demonstrated that the multiplication of V. cholerae was strongly temperature-dependent. No growth was observed at 10°C, 15°C, and 50°C. Moderate growth occurred at 25°C and optimum growth at 37°C as shows Table 2.
Growth at different pH levels indicated that V. cholerae thrives best in alkaline conditions. Maximum growth was observed at pH 11–12, whereas no growth occurred at pH 5. Limited growth was noted at pH 7, and moderate growth at pH 8 as shows at table 3.

Table 1. Biochemical and sugar fermentation tests of *Vibrio cholerae*.

Biochemical Tests	Result	Sugar Fermentation Tests	Result
Catalase	+	Sucrose	+
Voges Proskauer	+	Trehalose	+
Oxidase	+	Mannitol	+
Indole	+	Glucose	+
Citrate	+	Inositol	–
Urease	–	Sorbitol	–
Methyl Red	–	Lactose	–

Table - 2: Growth of *V. cholerae* on different temperatures

Sr/ No	Temperatures (°C)	Growth	Remarks
1	10	-	No growth
2	15	-	No growth
3	25	+	Moderate growth
4	37	+++	Optimal growth
5	50	-	No growth

Table - 3: Effects of pH on *Vibrio cholerae* growth

S. No	pH	Growth	Remarks
1	5	--	No growth
2	7	+	Less growth
3	8	++	Moderate growth
4	11	+++	Good growth
5	12	++++	Maximum growth

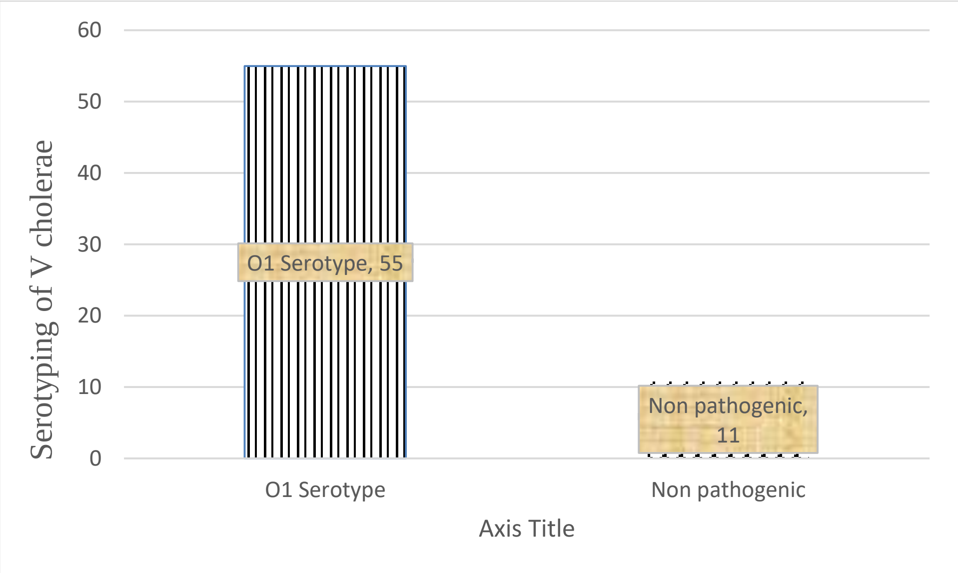

Figure-1: O1 serotype of *V. cholerae* and nonpathogenic

Discussion

Serological identification of Vibrio cholerae remains essential in distinguishing epidemic-causing serogroups such as O1 and O139 from non-O1/non-O139 strains, which are often linked with sporadic cases. Environmental factors play a crucial role in the survival and spread of the pathogen, with temperature and pH exerting significant influence on its growth. The organism shows optimal proliferation at moderate temperatures and thrives best in alkaline conditions, particularly between

pH 8 and 12. These characteristics explain its persistence in aquatic environments and its ability to trigger outbreaks under favorable ecological conditions. **Huq et al, 2005**
In the present study, Vibrio cholerae was isolated 7.5 % from suspected cholera patients across Balochistan. The diagnosis was confirmed through isolation and biochemical testing, and almost similar findings have also been reported by **Sreedhara HG et al. (2019)**.
When examined by Gram staining, the organism appeared as a Gram-negative curved rod. On TCBS agar, the isolates produced slightly flattened yellow colonies, a characteristic feature of Vibrio cholerae. These observations are in agreement with the report of **Sack et al. (2004)**, who also described V. cholerae isolated from stool samples as forming smooth to flattened yellow colonies, sometimes with a mucoid or translucent appearance.
All isolates obtained from stool samples of patients across different regions of Balochistan were subjected to various biochemical tests, including oxidase, catalase, motility, VP, indole, urease, and Simmons's citrate. These isolates tested positive for all the assays, and our findings are in agreement with the results reported by **Dua et al. (2017).**
In the present study, 66 isolates of Vibrio cholerae were analyzed, of which 55 belonged to serogroup O1 and 11were classified as non-O1/non-O139 serogroups. Within the O1 serogroup, all isolates were identified as the Ogawa serotype of the El Tor biotype. These findings are in line with the observations of **Rijal et al. (2019**), who also reported a predominance of O1 Ogawa El Tor strains in their study population. Such consistency across studies highlights the continued dominance of O1 El Tor Ogawa in cholera outbreaks, while non-O1/non-O139 strains persist sporadically as minor contributors.
Isolated organisms were observed at different temperatures in the current study from 15 to 50oC. The organism showed best growth at 37oC. However, growth was also seen at 20-40oC, but no growth was seen at 15oC, 45oC and 50oC. Our findings are in line with, **AL-Hadrawi and Ahmed, (2018),** who also evaluated the V. cholera growth at different temperatures and reported 30-37°C to be the best and optimal temperature for the growth of tested V. cholerae strains.
The pH trial showed maximum growth of V. cholerae between 8 -11 as compared with low pH. This observation is almost similar to the reported by **AL-Hadrawi and Ahmed, (2018)** findings.

Reference:

1. AL-Hadrawi HA, AL-Harmoosh RA. 2018. Effect of some chemical and physical factors on the growth of Vibrio cholerae isolated from patients in Najaf Province/Iraq. SCIREA Journal of Biology 3, 26-38.
2. Dua, p., Karmakar, a., Dutta, k., & Ghosh, C. (2017). A simple procedure for isolation, identification and characterization of Vibrio cholerae from clinical samples. International Journal of Pharma and Bio Sciences, 8(4), 57-64.
3. Harris JB, Khan AI, LaRocque RC. Blood group, immunity, and risk of infection with vibrio cholerae in an area of Endemicity. Infection and Immunity. 2005;73(11):7422-7427. DOI: 10.1128/IAI.73.11.7422-7427.2005.
4. Holmgren J, Lönnroth I, Svennerholm L. 1973. Fixation and inactivation of cholera toxin by GM1 ganglioside. Scandinavian Journal of Infectious Diseases 5, 77-78.
5. Huq A, Sack RB, Nizam A, Longini IM, Nair GB, Ali A, Morris Jr JG, Khan MH, Siddique AK, Yunus M, Albert MJ. Critical factors influencing the occurrence of Vibrio cholerae in the environment of Bangladesh. Applied and environmental microbiology. 2005 Aug;71(8):4645-54.
6. Igbinosa EO, Okoh AI. 2008. Emerging Vibrio species: an unending threat to public health in developing countries. Research in Microbiology 159, 495-506.
7. Kaper JHRS, Lockman H, Colwell RR, Joseph SW. 1979. Ecology, serology, and enterotoxin production of Vibrio cholerae in Chesapeake Bay. Applied and Environmental Microbiology 37, 91-103.
8. McCormack WM, DeWitt WE, Bailey PE, Morris GK, Soeharjono P, Gangarosa EJ. 1974. Evaluation of thiosulfate-citrate-bile salts-sucrose agar, a selective medium for the isolation of Vibrio cholerae and other pathogenic vibrios. Journal of Infectious Diseases 129, 497-500.

9. Rijal N, Acharya J, Adhikari S, Upadhaya BP, Shakya G, Kansakar P, Rajbhandari P. 2019. Changing epidemiology and antimicrobial resistance in Vibrio cholerae: AMR surveillance findings (2006–2016) from Nepal. BMC Infectious Diseases 19, 1-8.
10. Russell, A. D. (2003). Lethal effects of heat on bacterial physiology and structure. Science Progress, 86(1-2), 115-137.
11. Sack DA, Sack RB, Nair GB, Siddique AK. 2004. Cholera. Lancet 363, 223-233.
12. Sreedhara HG, Mohan NK. Molecular epidemiology of vibrio cholerae causing outbreaks and sporadic cholera in and around Hassan district and its antibiotic susceptibility pattern. IP International Journal of Medical Microbiology and Tropical Diseases. 2019; 5(1):41-6..
13. Weil, A., Midani, F., Chowdhury, F., Khan, A., Begum, Y., Charles, R., & Larocque, R. (2015). The gut microbiome and susceptibility to Vibrio cholerae infection. In Open Forum Infectious Diseases. Infectious Diseases Society of America 2 (1), 697).
14. Winn, W. C., Allen, S., Janda, W., Koneman, E., Procop, G., Schreckenberger, P., & Woods, G. (2005). Curved Gram-Negative Bacilli and Oxidase-Positive Fermenters: Campylobacteraceae and Vibrionaceae. In "Koneman's Color Atlas and Textbook of Diagnostic Microbiology. 6th Edition, Lippincott Williams and Wilkins, New York