Vibrio Cholera 22: Isolation And Molecular Detection of Cholera Toxin (CTX) in Some Iraqi Governorates

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***Abstract*— *Vibrio cholerae* is a major etiology of diarrhea in humans worldwide. In this study, we isolated and identified *V. cholerae* from the human stool of suspected cases admitted in different hospitals of Iraqi governerate.**

A total of (180) suspected patient stool samples infected with *Vibreo cholera* (*V. cholera*) ,110 sample were positive and showed growth of yellow colored colonies when cultured on Thiosulfate citrate bile salt agar(TCBS). The isolates were Gram-negative, curved shaped, and motile. Biochemically, they were found positive for indole and Methyl Red tests and negative for Voges–Proskauer test when we used API 20 test. Ninty of the isolates were ogawa when made serotyping test. Antibiotic sensitivity test revealed these isolates as highly sensitive to chloramphenicol, tetracycline m Ampicillinm , and Erythromycin while resistant to TR , and oxacillin. After the routine culture of samples for isolation and identification of *V. cholerae* isolates, PCR was performed for molecular detection of *V. cholerae* isolates based on 16S ribosomal RNA and ompW genes. Toxigenicity was detected by CTX toxin genes. Out of the 110 positive samples, only 50 isolates were positive for CTX gene, using genus-specific primers.

***Index Terms*—*A. eupatoria*, gastric ulcer, histology, endogenous enzymes.**

I. INTRODUCTION

A member of the Vibrioneceae family is the genus Vibrio. Vibrio are porous, gram-negative rods that can be straight or curved. When cultivated on liquid media, they are mostly motile via a single polar flagellum [1]. There are 72 species in this genus at the moment, 12 of which are found in clinical samples from humans [2]. Most human Vibrio infections are caused by V. cholerae, V. parahaemolyticus, and V. vulnificus among these 12 species [3]. As in the Enterobacteriaceae, V. cholerae has somatic (O) and flagellar (H) antigens, with the different O groups being referred to as serogroups or serovars V. cholerae O1 can be subdivided into three serotypes; Inaba (O antigens A and C), Ogawa (O antigens A and B) and Hikojima (O antigens A, B and C). *Vibrio cholera* is the most important species in the genus *Vibrio*. Diarrhea is a global problem and frequently affects children in Bangladesh due to the geographical location. World Health Organization (WHO) has identified the diarrheal illness as the second leading factor causing 760,000 deaths annually in children of less than 5 years old, of which 10% of the population from low- and middle-income countries, including Bangladesh [4] . Consumption of polluted water and food is a major contributing factor to the majority of human Vibrio diseases [5, 6]. Feeding uncooked or raw shrimp and shellfish can potentially result in a Vibrio infection [7, 8]. The transmission of intestinal infections like cholera has been linked to Bangladesh's dense population and inadequate personal and household cleanliness practices [9, 10]. When germs are introduced into areas with inadequate water and sanitation systems, Vibrio cholerae can spread swiftly . Although cholera requires a substantially greater infectious dosage of approximately 104 organisms, outbreaks are caused by their short incubation time (a few hours to five days for most subtypes) [11].. In order for people with cholera to recover, antimicrobial therapy is essential. Tetracycline and doxycycline resistance in Vibrio has quickly developed as a result of the widespread use of antibiotics as prophylactic during cholera outbreaks in Africa in the 1970s and 1980s, as well as in South America in the 1990s. The World Health Organization then advised against using widespread antibiotic use as cholera prophylaxis [12]. In many nations throughout the world, especially developing nations like Bangladesh, bacterial cholera resistance to antibiotics such as ampicillin, gentamicin, kanamycin, streptomycin, tetracycline, trimethoprim, and sulfonamides has become a major therapeutic issue [13].. Moreover, multidrug resistance in V. cholera serotype O1 biotype El Tor has been documented recently all over worldwide [14,15]. It is clear that the pathogenesis of cholera is, in fact, a very complicated process, involving more than just a few factors that propel the organism to reach the small intestine's epithelium and colonize it, producing CT (cholera enterotoxin), which prevents intestinal epithelial cells from conducting ion transport. However, the ctxAB gene on the 6.9-kb CTX prophage incorporated inside chromosome is still present in all strains of pandemic V. cholerae[16]. Conversely, the pathogenesis of V. cholerae is reliant on the generation of cholera toxin (CTX) and TCP (toxin-coregulated pilus). The cholera toxin basically functions as an adenosine diphosphate ribosylating toxin is consist of one A sub-unit (ctxA) that provides the intracellular activity, and five B subunits (ctxB) that binds holo-toxin to the cell receptor (17). The cholera toxin gene is located on a 4-5 kb DNA segment, and is flanked by two or more copies of a DR (direct repeat) sequence that varies

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in length from 2.4 to 2.7 kb. The presence of the ctxAB gene confirms the toxigenicity of V. cholerae. The pathogenesis of V. cholerae primarily depends on the production of TCP & CTX, and the biogenesis of toxin-coregulated pilus is dependent on the tcp operon, which consists of a large cluster of 12 genes. The identification of serogroups and virulence factors, along with the timely detection of V. cholerae infection in patients with diarrhea, is crucial for patient treatments and the control of the disease's spread. Molecular detection technology has made several PCR-based detection techniques possible and applied, including conventional PCR, real-time PCR, and multiplex PCR[18]

Materials and Methods

Ethical Approval: An informed agreement was achieved from each patient before their inclusion in the study.

Sample Collection

A total of 180 stool samples were randomly collected from cholera suspected patients of some governorates in Iraqi Hospitals.

Isolation and identification of Vibrio species From the stool samples, Vibrio species were isolated and tentatively identified using a culture method on Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) agar plates at 37°C for 18–24 hours aerobically. The isolates were then detected using API 20 for enterobacterace isolates and by staining and biochemical tests (Methyl red, Voges–Proskauer, indol, catalase, and oxidase tests). By cultivating each isolate on blood agar plates, hemolytic activities were also investigated, and the hanging drop method was used to measure motility [17].

Biochemical Identification

To inoculate an API 20E strip (Biomerieux Industries, France) with each isolate, one colony from an overnight culture on nutrient agar with 2% NaCl (NA2) was floated in sterile saline water (0.85% NaCl) in accordance with the manufacturer's instructions. After that, this was incubated for 24 hours at 37°C. The API 20E analytical profile index (version 5) was used to analyze the set of 21 miniature biochemical tests. Two statistical metrics (t value and % of identification [id]) were used to assess the quality of the identification. The identification results in this investigation were categorized as follows: (a) excellent identification when 90.0 ≥% of id <99.9% and t value ≥0.75; (b) good identification when t value ≥0.25 and % of id <99.9%; and (c) low discrimination, when V. cholerae was detected.

Serologic Identification of V. cholera O1 and O139 Presumptive identification using O1 and O139 antisera For slide agglutination testing with polyvalent O1 or O139 antisera. A nonselective agar medium containing fresh growth of suspected V. cholera should be utilized. The use of TCBS agar growth could lead to false-negative results. B. Growth on the slant's surface should typically be sufficient after 5 to 6 hours of incubation to perform slide serology using antisera; if not, incubate for an extended amount of time. C. Test with O139 antiserum if the isolate does not agglutinate in O1 antiserum. D. It may be reported as presumed V. cholera O1 or O139 if the polyvalent O1 or O139 antiserum test results are positive. E. Monovalent Ogawa and Inaba antisera should be used to evaluate presumed V. cholera O1 isolates. Use of Inaba and Ogawa antisera to confirm the presence of V. cholera O1 Important information A. Three serotypes of V. cholera have been identified within the O1 serogroup: Inaba, Ogawa, and the extremely rare Hikojima. B. Agglutination of monovalent antisera to type-specific O antigens provides the basis for serotype identification. C. The identification of a V. cholera O1 isolate can be verified by a positive reaction in either the Inaba or Ogawa antiserum. D. Serogroup O1 is not thought to include isolates that agglutinate slowly or weakly with serogroup O1 antiserum but not with Inaba or Ogawa antiserum. Test for antibiotic sensitivity.

Antimicrobial Susceptibility Testing

Nine commonly used antibiotics (HiMedia, India), namely, azithromycin (15 mg), chloramphenicol (30 mg), ciprofloxacin (5 mg), erythromycin (15 mg), gentamicin (10 mg), norfloxacin (10 mg), oxacillin (15 mg), streptomycin (10 mg), and tetracycline (30 mg) were selected for the sensitivity test. Antibiogram profile of the isolates was determined by the disk diffusion method on Mueller Hinton (HiMedia, India) agar plates, as described by Bauer et al. [19]. McFarland 0.5 standard was maintained for each culture suspension of bacterial isolates before the antibiogram study. As per the recommendations of CLSI [20], the results of the antibiogram were recorded as sensitive, intermediately sensitive, or resistant.

Molecular Detection Of V. Cholerae

Molecular detection of the suspected *Vibrio* isolates at the genus and species level was carried out by PCR using two sets of designed primers targeting 16 s rRNA and ompW genes, one at genus level and another at species level. DNA from pure broth culture was extracted by using specific kit. The PCR reactions were done in an in a 25 µl reaction scale with 12.5 µl master mixture 2X (Transgene-China). Amplified products were analyzed by electrophoresis in 1.5% agarose gel. Amplified products were stained using ethidium bromide and finally visualized under ultraviolet trans-illuminator (Labent, USA).

-DNA extraction: DNA extraction was done on overnight subculture on NA_2 using the chloroform-phenol procedure . DNA purification of *V. cholerae* isolates were performed according to the genomic DNA purification protocol supplied by the manufacturing company(trans gene,China).

PCR assays: PCR amplification of the target DNA was carried out in an Eppendorf Thermocycler (Eppendorf, USA (using 200-µL PCR tubes with a reaction mixture volume of 25 µL containing 3 µL of template DNA, 0.2 µL of each primer (100 µM), 2.5 µL of 2 mM dNTP, 0.125 µL (5 U/µL) of Taq polymerase (Eurobio), 2.5 µL of 10X reaction buffer, 1.25 µL of $MgCl_2$ 50 M (Eurobio), and ultra-pure water. The sequences of the primers and the conditions of amplification used in this study are shown in Table 1.

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TABLE 1, PCR PRIMER SEQUENCES AND PRODUCT SIZE

Genes	Forward primer (5′--->3′)	Reverse primer(3′ --->5′)	Product size
V.coleria 16S rRNA 27F	TCTGAGACAGGTGC TGCATG 400	GCTTCTTTTGC AGCCCACTC	**400 bp**
V. cholerae ompW	**CACCAAGAAGGTGACT TTATTGTG**	**GGTTTGTCGAA TTAGCTTCACC**	304 bp
Cholera toxin ctxA,	**CGGGCAGATTCTA GACCTCCTG**		**565 bp**

The conditions of PCR included one cycle at 95°C for 5 minutes, then 30 cycles at 95°C for 30 seconds; the annealing included 30 cycles for 30 seconds at 58°C for the 16S rRNA gene , 62°C for ompW gene and 65°C for ctxA gene . Then conditions included 30 cycles at 72°C for 1 minute and one cycle at 72°C for 5 minutes; the final product was kept at 4°C. Agarose gel electrophoresis was used to confirm the successful PCR amplification. A UV transilluminator was used for the observation of DNA bands.

RESULTS

Isolation And Identification

Atotal of (180) suspected stool patients sample infected with Vibreo ,110 sample were positive for Vibrio and showed growth of yellow colored colonies when cultured on Thiosulfate citrate bile salt agar(TCBS)(fig. 1). The isolates were Gram-negative, curved shaped, and motile. The rest of the isolates were Gram-negative bacteria and are not included in the current study.

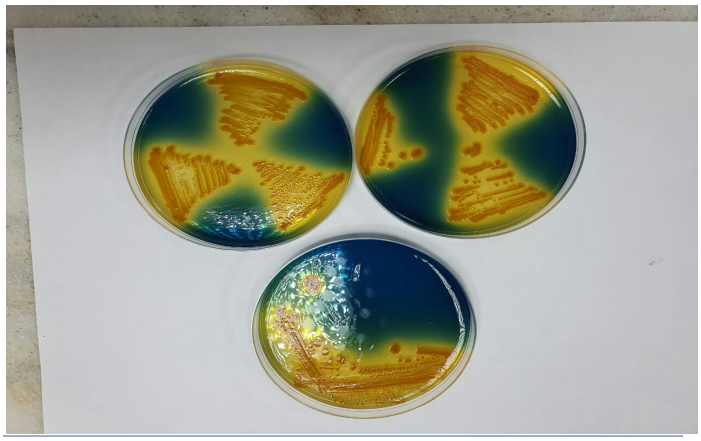

Figure 1 Growth of bacteria on TCBS agar medium.

Antibiotic sensitivity test revealed these isolates as highly sensitive to chloramphenicol, tetracycline m Ampicillinm , and Erythromycin while resistant to TR , and oxacillin.(fig. 2)

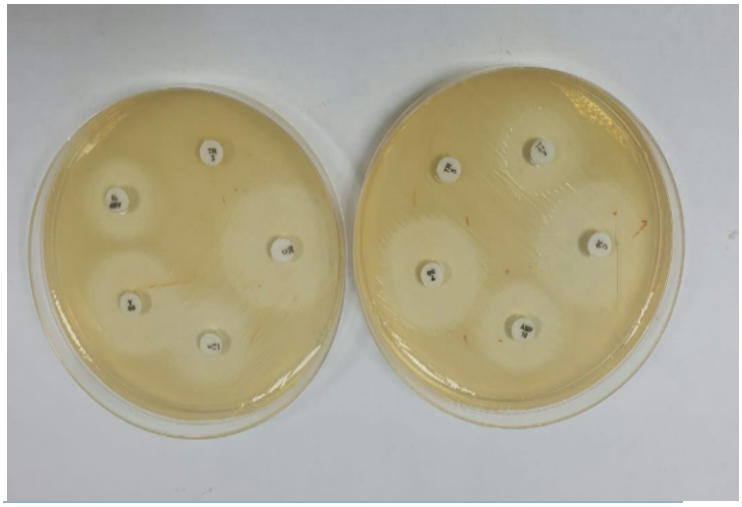

Figure 2 Antibiotic sensitivity test .

Biochemically, they were found positive for indole and Methyl Red tests and negative for Voges–Proskauer test when we used API 20 test. All the isolates were ogawa when we made serotyping test . *(fig. 3)f*

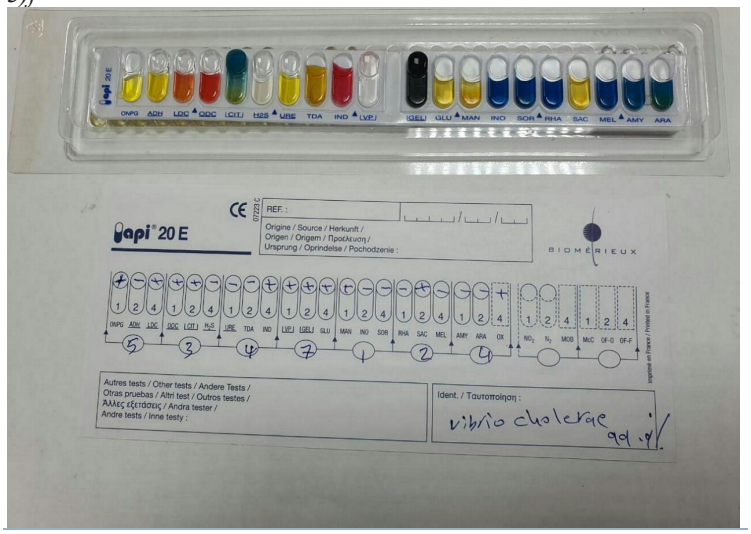

Figure 3 Biochemical Test.

Molecular Assay

Three primers were used to investigate the presence of three important genes these are V.coleria 16S rRNA 27 , V. cholerae ompW and Cholera toxin ctxA genes for V. cholera isolated from 180 stool samples. It was found that V.choleria 16S rRNA 27 is present in all the 110 isolates, with molecular length in (400 bp) as was shown in Fig. (4-A) Molecular detection of ompW gene was observed in (90) isolates of V. cholerae with molecular length in (304 bp) as shown in Figure (4-B). This study found that (50) isolates of V. cholerae gave positive amplicon ctxA gene with molecular length (bp) as shown in figure (4-C).

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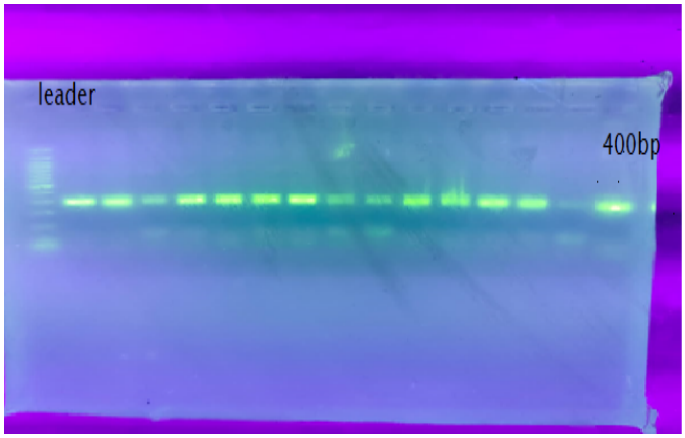
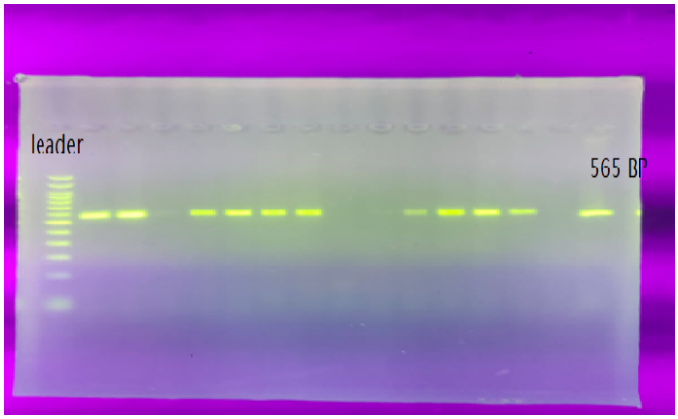


(Figure 4-A) Agarose gel electrophoresis for PCR product of 16S ribosomal RNA gene for confirmatory molecular detection of Vibrio cholerae at product size 400 bp



(Figure 4-c)Agarose gel electrophoresis for PCR product of ctx A gene for confirmatory molecular detection of Vibrio cholerae at product size 565 bp.

DISCUSSION

One of the etiologic agents is Vibrio cholerae. It is a bent, motile, noninvasive rod that belongs to the Vibrionaceae family and is gram-negative. The pathogenesis of V. cholerae is complex and involves both toxic action and virulence factors. The diarrheal condition associated with cholera is caused by the production of cholera toxins following the microorganism's colonization of the human gut. Furthermore, additional toxins that contribute significantly to the pathogenesis include ctxA (21).

The majority of individuals who live in Iraq's governed rural and urban areas do not have access to sanitary conditions [5]. The disease cholera is primarily linked to inadequate sanitation. In IRAQ, diarrheal illness is recognized as a primary contributor to infant death. One of the main causes of fatal diarrhea is Vibrio cholerae. The goal of the current investigation was to identify and isolate V. cholerae from feces samples that were thought to be human. Vibrio spp. were cultured and isolated using TCBS, a selective medium that is specific to Vibrio species. Similar to the findings of Choopun et al. [22, 23], one handred ten out of 180 samples were observed to form yellow color button-shaped flattened colonies on TCBS agar. Each isolate formed hemolytic colonies on blood agar, indicating the possibility of infection. On blood agar, all the isolates produced hemolytic colonies suggesting their ability to produce infection. The isolated Vibrio spp. was observed as motile also reported by Kaper et al. [24].

The ability of the target bacteria to be selected and the selection of a few pertinent taxonomic features for presumed identification are key components of routine isolate screening on any selective agar. It is often advised to use TCBS agar for Vibrio spp. isolation (11). Because of its superior capacity to separate vibrios from their native estuarine habitat, it eradicates species that are not tolerant of bile salts (6). Our findings support the suggestion made by Muic et al. (7) that any suggested trait selection for good presumed identification of vibrios would most likely require the use of TCBS agar as a selective medium. Selecting a confirmatory identification technique.

The API 20E system is indeed considered an acceptable method for the identification of the more commonly-occurring members of the family Vibrionaceae (10, 11), even if there are very few reports expressly concerned with the ability of commercial systems to identify members of the genus Vibrio (12)

The precise aetiologic agent of any epidemic must be identified through the molecular identification of pathogenic bacteria down to the species level. By employing PCR, researchers may distinguish between different isolates of V. cholerae without having to deal with the possible challenges that come with using traditional identification methods. Therefore, in order to identify Vibrio species specifically, the 16S rRNA gene has been required. For the molecular diagnosis of V. cholerae, a positive PCR result for the 16S rRNA gene was reported in 110 isolates. This result is consistent with several investigations that identified Vibrio spp. using the 16S rRNA gene. The precise aetiologic agent of any epidemic must be identified through the molecular identification of pathogenic bacteria down to the species level. According toTable 2,. Despite having rtx genes, it's possible that conventional testing missed the isolates' expression of cholera toxins. A study conducted in Hong Kong from 1986 to 1999 on isolates obtained revealed that the rtx gene was present in all 166 V. cholerae isolates. This gene is associated with V. cholerae's capacity to exhibit cytotoxic activity and phenotypic expression in every isolate under investigation. (21).

According to a study by Chatterjee et al., these RTX toxins have a significant role in the pathophysiology of V. cholerae. Previous studies have revealed that a high rate of V. cholerae isolated from the environment has the rtx gene clust[25].

High proportions of the V. cholerae genes rtxA (83.0%), rtxB (97.0%), rtxC (95.8%), and rtxD (95.5%) were discovered by others in freshwater fish isolates. According to related research, V. cholerae can live in the environment for an extended period of time, which explains why seasonal variations in temperature and climate as well as algal blooms are linked to cholera epidemics in Iraq [26].

In Iraq, many factors must be taken into consideration while studying the spread of cholera, including the source of the water supply, socioeconomic status, living conditions, high population density, poor hygiene, poor food safety, cultural

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beliefs and practices, levels of education, status of the immune system, age and gender. Interestingly, a brief description of the effects of these factors is provided in an Iraqi study by Karim and Darwish [27], who concluded that the outbreak in Iraq during 2017 was cholera associated with factors like being female, being 15 to 45 years old and living in a highly populated residential area with poor sanitation systems and a bad source of water (tap water, or reverse osmosis–treated water provided from local portable distributors).

Conclusion

Our detection of the 16S rRNA gene provided valuable information about the diagnosis of *Vibrio cholerae* isolates during the 2022 cholera outbreak in Iraq. This in turn further indicates the power of the currently utilized 16S rRNA–based primers to discriminate among *V. cholerae* species

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