Genotyping of *Vibrio cholera* for Virulence Factors in Diwaniyah City - Iraq

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Cholera is one of the most important epidemic diseases globally. It is causing of morbidity and mortality in the world. Severe watery diarrhea causes a composite process involving several component that help them reach the lining of the small intestine, form colonies and generate bacterial toxins. This survey was carried out to discover the genetic patterns of clinical isolates according to the presence or absence of toxic genes for the city of Diwaniyah in southern Iraq. Sixty isolates were isolated from patients with cholera. The isolates included the center of the city and the surrounding rural areas. Biochemical and serological diagnosis. All the isolates were *V. cholera* serogroup O 1 of the serotyping Ogawa in biotype El Tor. Genetic testing was carried out using PCR technique and base on the presence or absence of toxin genes. Three genotypes were identified for the region.

**Keywords:** *Vibrio cholera*, biochemical and surgical diagnosis.

One of the epidemic disease is cholera. Acute watery diarrhea disease is causing common in Iraq. There are several region of Africa, Latin America and south of Asia where seasonal outbreaks happen (Neelam, et al. 2012) And It was public health problem (Shears, 2001). *V. cholera* belong to the family *Vibrionaceae* which have 104 species (DSMZ, Leibniz). The strains of genus *V. cholera* classified to serogroups according to epitopic different for lipopolysacharide for surface of cell (Yamai et al., 1997) The serogroup O 1 and O 139 among 210 that causal agent for cholera (Revera, et al., 2002).

There are two distinct biotypes for O1 are classical and El Tor, every one of biotype has three serotypes are Ogawa, Inaba and Hokojima. (Nair, 2001).

Globally, the *V. cholera* have seven pandemics. Indian was originated for the first six pandemics of cholera via classical type for *v. cholera* O1. The seventh pandemic was El Tor that spread to the world which originated from Sulawesi in Indonesia. (Carlos and Eduardo, 1996)

Iraq is confronting tremendous calamities of obliteration of the foundations with lack of sterilized water of drinking particularly in poor areas and exile camp that aide for the presence of contaminate water with cholera. (Al-Abbasi, et al., 2005)

In Iraq, 1999 reported the first occurrence of O 139 in Baghdad although few cases. (Al-Abbasi et al., 2005). After war of Iraq in 2003 with program of the communicable disease reported cholera epidemics in many region of Basrah (Valencino et al., 2003). Biotype El Tor of serogroup O1 include serotype Inaba isolated from 4,667 cases in Iraq when outbreak epidemic cholera in 2007. (Khwaif et al., 2010). As well as,
Ogawa serotype reported in this epidemic (Saleh, et al. 2011)

All governorates in Iraq isolate Vibrio cholera O1 serotype Inaba with few cases only of Ogawa serotype form 2013 to 2015. (Al-Abbassi et al. 2015)

The enterotoxin (CT) of cholera which codes by ctxAB gene is causing the severity of disease (Joseph, et al. 2015). Strains O1 and O139 of V. cholera possess the ctxAB gene on the 6.9-kb CTX prophage integrated within chromosome which responsible for encoding enterotoxins. These Enterotoxins in V. cholera cause diarrhea. And ace gene coding for accessory cholera enterotoxin as well as zot coding for zonula occludens toxin.

The aim of present study to determined genotypes of clinical isolates of V. cholera according to presence and absence of enterotoxin genes are ompW, ctxA, ace, zot by using PCR technique for outbreak and sporadic cholera in Diwaniyah city.

MATERIAL AND METHODS

Specimen collection
In all, 60 isolates of V. cholera O1 were obtained in 2016 from patients having acute watery diarrhea. The samples were collected from Al Diwaniyah hospital in Diwaniyah city of Iraq.

These isolates were identified V. cholera O1 after culture and isolation (following World Health Organization 2014 guidelines) on TCBS agar (BIOMARK, Laboratories, India) ‘media via usual biochemical tests that included those for the fermentation of sugars in triple sugar iron agar medium, H2S and gas, indole formation, mannitol motility, oxidase, urease production, catalase production, nitrate reductase. Then isolates were confirmed by serology with commercially available antisera (Denka Seiken, Gosen-shi, Nilgata, Japan). Culture (18–24 h old) for these strains with specific density (0.5–0.63 McFarland standard, measured with colorimeter supplied with the system) and inoculate into sterile normal saline (supplied by BioMerieux, Lyon, France) then load on to Vitek 2 Compact system has software

| Table 1. Primers used in PCR* |
|-----------------------------|
| Gene | Sequence of nucleotides (5’-3’) | PCR product Size (bp) |
|ompW - F | 5’- CAACCAAGAAGGTGACTTTATTGTG-3’ | 588 |
|ompW - R | 5’- GAACCTATAACACCGCGTGA3’ | |
|ctxA - F | 5’- CGGGCAGATTCTAGACCTCCTG-3’ | 564 |
|ctx – R | 5’- CGATGATCTTGGAGCATTCCCAC-3’ | |
|ace - F | 5’- TAAGGATGTGCTTATGATGGAC-3’ | 316 |
|ace – R | 5’- CTGTGATGAAATAAGATACCTCATACG-3’ | |
|zot – A | 5’- TCGCTTAACGATGCGGCGT-3’ | 947 |
|zot - R | 5’- AACCACGTCTCCTCTACCCA-3’ | |

| Table 2. Results of vibrio cholera genes |
|----------------------------------------|
| Genes | No. tested | No. positive | Positive % |
|ompW gene | 60 | 60 | 100 |
|ctxA gene | 60 | 60 | 100 |
|Ace gene | 60 | 36 | 60 |
|Zot gene | 60 | 57 | 95 |

| Table 3. Genotypes of the Isolates |
|-----------------------------------|
| Grouping of genotype | CtxA | Ace | Zot | No.isolates | % |
|Genotype 1 | + | + | + | 36 | 60% |
|Genotype 2 | + | - | + | 21 | 35% |
|Genotype 3 | + | + | - | 3 | 5% |
|Total | | | | 60 | 100% |
version 4.01 (BioMerieux, Durham, NC) following manufacturer’s instructions for identification using GN (BioMerieux) cards. The isolates were also sent to the Central Health Laboratory of Cholera and Enteric Diseases, Baghdad, which be the WHO Collaborating Centre for research on diarrheal diseases to identify for these isolates.

**PCR test**

Genomic DNA for V. cholerae extracted carried out by geniod kit (Geniad). Then amplification the target DNA by PCR in a thermal cycler (Techne TC-3000, USA) was performed in a reaction mixture tube which add to it 1 µl for each primer “(forward and reverse),5 µl from gDNA extract and add dionized water to complete the final volume 20 µl.”

The primers were used for cholera toxin in this study (ompW) (ctxA), (ace), (zot). All primers were provided by (Bioneer company, Korea) showing in (Table 1). The program of thermocycler was as follows: preincubation at 95°C for 2 min, 30 cycles for 30 sec. at 95C for denaturation, 1 min at 64.8°C for annealing, 30 sec. at (57.8, 62.9 and 59.9 for ompW, ctxA and zot respectively)C for elongation and incubation at 72C for 5 min for final elongation. This protocol for each ompW, ctxA and zot genes. While the protocol for ace gene was touchdown PCR protocol. It was performed on 45 cycles of predenaturation at 95°C for 2 min, followed by the 15 cycles with successive annealing temperature decrements that 0.5°C changed from 63°C to 56.5°C in every cycle versus time increments (30 sec in the first 15 cycles and 30 sec in the last 20 cycles). The reaction was denatured at 95°C for 30 min, followed by annealing at this temperature algorithm and polymerization at 72°C for 40 sec. Subsequently, the 20 cycles of denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec, and elongation at 72°C for 40 sec were employed. The last primer extension step was done at 72°C for 5 min. Amplicons were made electrophoresis in agarose 1.5% gel. (Appligene, Illkirch, France) then stained with ethidium bromide (5mg/ml) for visualized.

![Fig. 1. Agarose (1.5%) gel electrophoresis of ompW gene with 588 bp in lanes (1-20). M was DNA marker](image1)

![Fig. 2. Agarose (1.5%) gel electrophoresis of ctxA gene with 564 bp in lanes (1-20). M was DNA marker](image2)
RESULTS

Biotyping and serotyping

A sixty isolates of *V. cholera* were examined by serogroup, serotyping and biotyping. All isolates were diagnosed *V. cholera* serogroup O1 of serotyping Ogawa in biotype El Tor.

Genotyping

Genotypically, the description of isolates were shown in table(2). All *V. cholera* O1 isolates were positive for the species specific gene *ompW*. The gene of toxin *ctxA* was found in all isolates. And ace gene showed 36(60%) of the isolate while 24(40%) was negative for this gene. Fifty seven of isolates (95%) was positive for Zot gene and 3 (5%) was negative . For study genotypes for isolates grouped isolate to three isolate based on presence and absence toxins of cholera table - 3.

DISCUSSION

The larger part of *V. cholerae* strain in nature are thought its innocuous estuarine microbes. Nonetheless, particular strains seem to be advanced that reason sickness for people by successfully colonize in small digestive system and discharging a powerful enterotoxin . Four genes in chromosomal DNA codes for destructiveness related factors in *V. cholerae* (*OmpW, ctxA, ace* and *zot*) were incorporated into this investigation.

It found all isolate positive for Omp gene. So that indicate all samples were *V. cholera*. The strains isolated from the common source are important for epidemiology, through which the genetic relationship of the isolates is determined and determines the source of transmission, spread and origin of cholera infection. In previous studies demonstrated that *omp W* gene represented as inner control for testing *V. cholera* Goel, *et al*. 2007; Goel, *et al*. 2010; Izumiya, *et al*. 2011; Jain,* et al*. 2011. Our results agreement with Menezes *et al*. I concerned that identification of *v.cholera* via *ompW* gene give result 100% (Menezes *et al*. 2014).

The genetic elements of CTX has a compound transposon with 4.5 kb central core

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![Fig. 3. Agarose (1.5%) gel electrophoresis of ace gene with 316 bp in lanes (5,7,9,10,11,12,15,16,17,18and 19). M was DNA marker](attachment:image3.png)

![Fig. 4. Agarose (1.5%) gel electrophoresis of zot gene with 947 bp in lanes(1-20 except lane 6 ). M was DNA marker](attachment:image4.png)
region (ctx AB, zot, ace, orfU, and cep) which encodes for both CT and functions for virion and flanked with one or more copies of a 2.7 Kb repetitive sequences that responsible for regulation, replication and integration of ctxö " (Rivera et al. 2001). we tested three genes in this region (ctxA, ace, and zot) in our isolates.

Enterotoxin gene ctx A found in all isolates. This gene is causative agent to severe diarrhea. All our isolates are Toxigenic strains because possessing ctx elements (Julianai et al., 2000; Chen et al., 2004). And ctxA gene located in this element (Waldor et al., 1996). This result agree with Parrick et al., 2012 and Rivera et al., 2001 that their result found ctxA gene was 100% in their toxigenic isolates. Ratnam et al. show 98% for their isolate of V. cholera that ctxA gene was positive for ctx gene in their clinical isolate (Ratnam et al. 2015). However, Menezes et al. show negative results when tested environmental samples via ctxAB gene. Other studies not found positive tested for ctxA gene in their isolates Gonçalves et al. 2004. While THEOPHILO et al. found one positive isolate for virulence factor ctxA gene in their clinical isolate (Ratnam et al. 2015).

Accessory cholera enterotoxin (ace) and (zot) gene show high percentage which was 60% and 95% in all our isolates. In contrast Poursahie et al. found lower percentage for virulence cassette genes ( Poursahie et al. 2002). The zot toxin is increases permeability of the gut via intercellular tight junction or (zonula occludens) (Fasno et al., 1999).

However, in all our result show 60% of V. cholera has three genes ctx, ace and zot in genotype 1. In genotype 2 show presence ctx, zot genes and absence ace gene was 35% for all isolates of V. cholera. However, Rivera et al. found that ctxA and zot genes in their toxigenic isolates was 100%. (Revira et al. 2001). Third genotype show presence ctx, ace genes and absence zot gene was 5% for all isolates of V. cholera that result agree with many studies (Chowdhury et al., 1995; Ghosh et al., 1997; Karasawa et al.), 1993).

Cholera is still a major epidemic disease that must be investigated and deepened. The continuous monitoring of cholera strains should be carried out through genomic research and the investigation of new strains. Despite the development of medical services. Precautions must be taken to prevent infection. Therefore, the clones were determined their genotype according to virulence factors and resistance to antibiotic.

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