

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 lipopolysaccharide 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 all.*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,

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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

Tables 1. Primers used in PCR*

Gene	Sequence of nucleotides (5'-3')	PCR product Size (bp)
<i>ompW</i> - F	5'- CACCAAGAAGGTGACTTTATTGTG-3'	588
<i>ompW</i> - R	5'- GAAGTTATAACCACCCGCG3'	
<i>ctxA</i> - F	5'- CGGGCAG ATTCTAGACCTCCTG-3'	564
<i>ctx</i> - R	5'- CGATGATCTTGGAGCATTCC CAC-3'	
<i>ace</i> - F	5'- TAAGGATGTGCTTATGATGGACACCC-3'	316
<i>ace</i> - R	5'- CGTGATGAATAAAGATACTCATAGG-3'	
<i>zot</i> - A	5'- TCGCTTAACGATGGCGCGTTTT-3'	947
<i>zot</i> - R	5'- AACCCCGTTTCACTTCTACCCA-3'	

Table 2. Results of *vibrio cholera* genes

Genes	No. tested	No. positive	Positive %
<i>ompW</i> gene	60	60	100
<i>ctxA</i> gene	60	60	100
<i>Ace</i> gene	60	36	60
<i>Zot</i> gene	60	57	95

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 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, Bagdad , which be the WHO Collaborating Centre for research on diarrheal diseases to identity for these isolates.

PCR test

Genomic DNA for *V.colera* 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 weres 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 64.8C 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

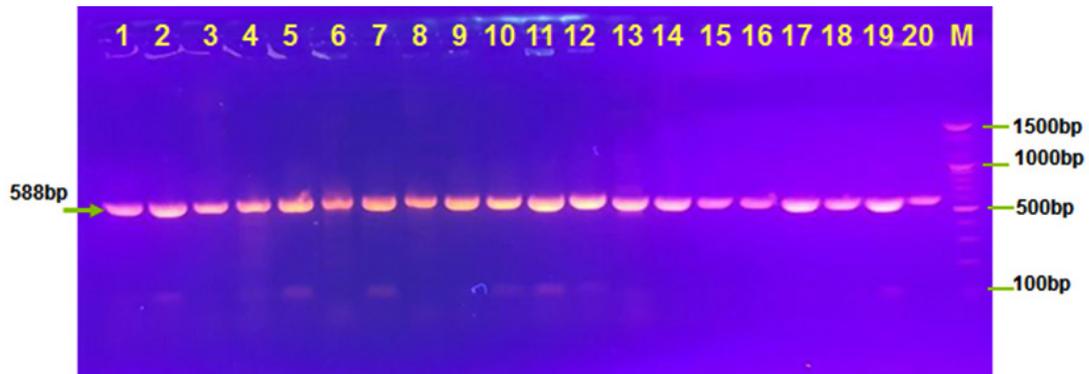


Fig. 1. Agarose (1.5%) gel electrophoresis of ompW gene with 588 bp in lanes (1-20) . M was DNA marker



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

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.* 1 concerned that identification of *v.cholerae* 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

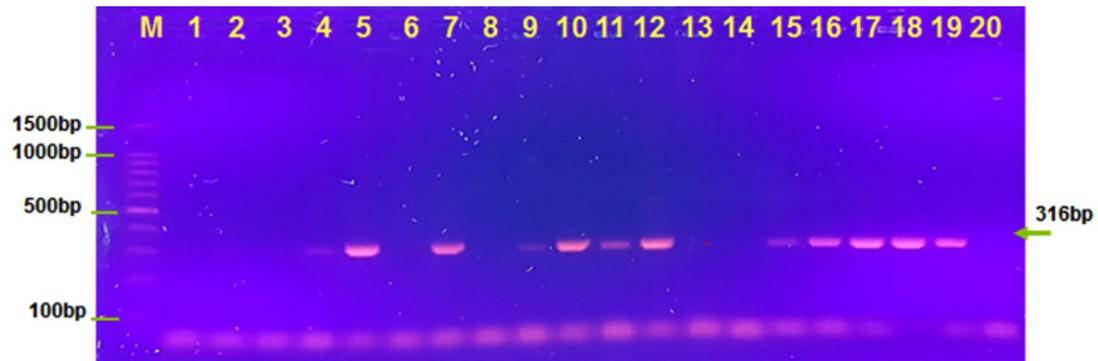


Fig. 3. Agarose (1.5%) gel electrophoresis of *ace* gene with 316 bp in lanes (5,7,9,10,11,12,15,16,17,18 and 19). M was DNA marker

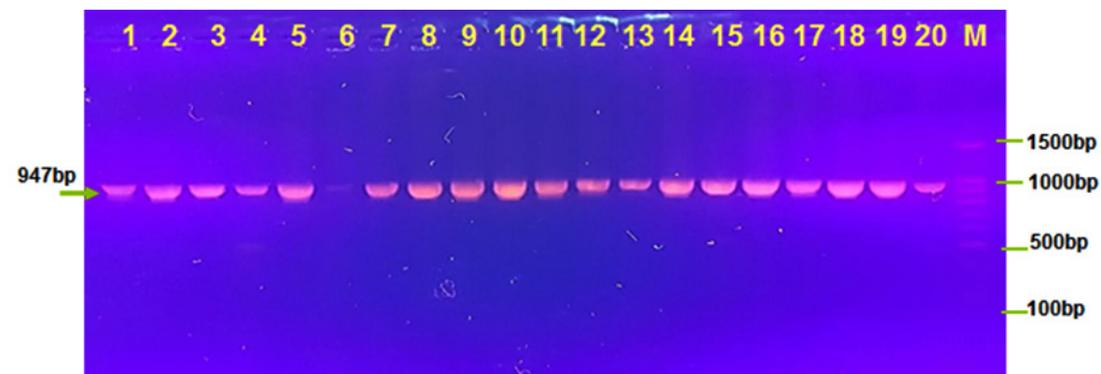


Fig. 4. Agarose (1.5%) gel electrophoresis of *zot* gene with 947 bp in lanes(1-20 except lane 6). M was DNA marker

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 2.7Kb 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 caustive agent to sever diarrhea. All our isolates are Toxogenic 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 Riveraa *et al.*,2001that their result found ctx A gene was 100% in your toxigenic isolates. Ratnam *et al.* show 98% for their isolation of *V. cholera* 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 Gonçaves,*et al.* 2004. While THEOPHILO *et al* found one positive isolate for virulence factor ctx A gene in their environmental strains of non O1 and O139 Theophilo,*et al.* 2006.

Accessory cholera enterotoxin (ace) gene and (zot) gene show high percentage which was 60% and 95% in all our isolates .In contrast Pourshafie *et al* found lower percentage for virulence cassette genes (Pourshafie *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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