Molecular Detection of Cholera Infection during the Outbreak in Thi-Qar Province /Iraq in 2015-2016

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Abstract: Cholera is an acute disease caused by Vibrio cholerae; it's affected to all aged groups. Cholera infection is outbreaks in Iraq as reported for several years. The recent cholera outbreak, emerged throughout 2015-2016, was investigated by using bacteriological laboratory tests, singleplex and multiplex PCR technique for the detection of V. cholerae from stool samples. Furthermore the antibiotic susceptibility test for cholera was also investigated coupled with the toxigenic potential. A total of Twenty Vibrio cholerae isolates were isolated from diarrheal patients in Thi-Qar province. These isolates were diagnosis by conventional biochemical test, API20 E system and molecular methods by using 16SrRNA. The isolates were characterized for gene traits; antimicrobial susceptibility . The results appeared all 20 isolates were positive for 16S rRNA. Multiplex PCR analysis revealed that 65%, 35% and 15% of isolates were positive for tox R, tcp and ctx B genes respectively .The antimicrobial susceptibility testing to isolates revealed high levels of resistance to ampicillin (100%), nalidixic acid (90%), sulfamethoxazole-trimethoprim (80%), tetracycline and ciprofloxacin (55%) and chloramphenicol (45%) in addition to increase the prevalence of multidrug resistant (MDR) between Vibrio cholerae isolates.

1 Introduction

Cholera is an acute disease caused by the bacteria Vibrio cholerae bacterium, is a continuous public health problem in poor socio-economic conditions countries, Cholera has the potential to appear in explosive outbreak, epidemic or even pandemics [1]. Vibrio cholerae is the aquatic bacterium responsible for the cholera disease, it is noninvasive, gram-negative bacterium and it is responsible for severe epidemics of cholera and endemic diarrhea in many countries, especially the development countries [2].V. cholerae is able of infecting humans, resulting the loss of fluids in the form of watery diarrhea and if left untreated frequently leading to death due to dehydration. The human intestine provides the better conditions to the expression of virulence factors and allows the organisms to colonize and cause disease then prepare for re-entry into the environments, V. cholerae pathogenesis involves infection, reaching and colonizing in the epithelium parts of the small intestine. Within the epithelium intestine, where V. cholerae express a variety virulence factors ( extracellular and aggressive) .The two main virulence factors are toxin-coregulated pilus (TCP) and cholera toxin (CT) ;both are essential for the colonization of the host and enterotoxicity, respectively [3] [4]. V. cholerae virulence cascade toxR protein of regulated the expression of several virulence factors including TCP that playing essential roles in V. cholerae pathogenesis [5]. There are at least six virulence genes in V. cholerae; these genes regardless as regulator factors
and they are: toxR, ctxA, tcpA, OmpW, ace, zot and rfbO1; which may commonly be found in all V. cholerae serotypes [6] [7]. The tcp genes are located on the Vibrio pathogenicity island (VPI). CT is an ADP-ribosylating toxin composed of two subunits that causes an increase in cAMP in intestinal cells, leading to diarrhea due to the osmotic imbalance. People infected with cholera may suffer from different clinical symptoms including vomiting, fever, hypotension, removed important body fluids, salts and vital nutrients. They may also suffering from body spasm, paralysis and fainting [8] [9]. In the last two decade; cholera disease in Iraq is represented as an epidemics infection This has worsened especially after the first Gulf war in 1991 when cholera disease became endemic in all provinces of Iraq [10] [11]. From 2007 to 2009 many Iraqi people have been suffering from the cholera disease [12]. The antibiotics are commonly used for treatment of severe cases to reduce the duration of cholera, the rapid emergence and spread of multidrug resistant V. cholerae with resulting outbreaks across the globe can undermine the success of antimicrobial therapy [13]. During the epidemics there is a great variation in patterns of antibiotic resistance at different times and different places, with multiple antibiotic-resistant V. cholerae commonly found. The other factor contributing to the emergence of antimicrobial drug resistance in V. cholerae include an increase in the number of susceptible people in communities, international travel and movements of population, and the breakdown of public health measures [13]. This study, we report molecular characterization of twenty V. cholerae isolates from patients during a cholera outbreak in in Thi Qar province during 2015-2016 and characterized for antimicrobial susceptibility on V. cholerae isolates. This study contributes to our understanding of cholera infection and the evolution of V. cholerae pathogenicity.

2 Materials and methods

Isolation and identification of V. cholerae

2.1 Samples collection

Three hundred fecal samples were collected from patients suffering from watery diarrhea, from both sexes in Thi-Qar province during period between August 2015 and March 2016. Fecal samples were put immediately in sterile tube contained alkaline peptone water.

2.2 Bacterial cultures

Alkaline peptone water with stool mixing incubated at 37°C (6-8 hours) then spread a 10 μl loop full from the inoculated and incubated alkaline peptone water on TCBS and on MacConkey agar plates and Blood agar then incubate at 37°C overnight (18-24 hours) and read the plates. V. cholerae suspected colonies on TCBS and on MacConkey agar plates and Blood agar onto non-selective media, (nutrient agar) plates for biochemical confirmation of V. cholerae [14].

2.3 Biochemical tests

The important biochemical tests were achieved according to Winn et al., 2007[15]. The tests are {Kligler iron (KI), Oxidase test, Urease test, Indole test, Citrate utilization test}.

2.4 Api-20E system (Analytical profile index for Enterobacteriaceae test)

Api-20E system is used clinically for the rapid identification of the V. cholerae isolates this test done according to Leboffe and Piercr, 2005 [16].

2.5 Detection of gene traits

2.5.1 Extraction of genomic DNA from bacterial culture

Genomic DNA was extracted from V. cholerae isolates by using Geneaid Genomic DNA Purification Kit (UK) and done according to company instructions; V. cholerae culture has been inoculated in 10 ml LF broth medium and incubated at 37°C overnight in shaking incubator.

2.5.2 Estimation of DNA Concentration

The extracted genomic DNA is checked by using Nanodrop spectrophotometer which measures DNA concentration (ng/µl) and checks the DNA purity by reading the absorbance at (260 /280 nm) [17].

2.5.3 Molecular diagnosis of V. cholerae isolates

The composition of the PCR mixture was prepared in total volume 20µl for 16 S rRNA gene which done as in table (1).Amplification of 16 SrRNA gene was achieved on the Thermo cycler as in table (1). The PCR products were visualized by electrophoresis on 1.2% agarose gels in 1X TBE buffer at 50 V for 85 min.

Table 1: primers used in PCR for detection of 16 S rRNA in V. cholerae

| Gene Name | DNA Sequences (5'-3') | Product Size (bp) | References |
|-----------|----------------------|------------------|------------|

2
3

Table 2: PCR condition of 16S rRNA in V. cholera

| St. No. | Step            | Temperature ºC | Time   | Number of Cycles |
|---------|-----------------|----------------|--------|-----------------|
| I       | Initial Denaturation | 96             | 5 min  | 1               |
|         | Denaturation      | 94             | 30s    |                 |
| II      | II Annealing      | 63             | 30s    | 30              |
|         | III Extension     | 72             | 30s    |                 |
| III     | Final Extension   | 72             | 7 min  | 1               |

2.5.4 PCR detection of virulence genes

All V. cholerae isolates were screened for three virulence genes (tcp, toxR and ctxB) by a multiplex PCR method (Skyberg et al., 2006). The Primers used for our study are listed in table (3). Amplification of virulence genes were achieved on the Thermo cycler as in table (4). The PCR products were visualized by electrophoresis on 1.2% agarose gels in 1X TBE buffer at 50 V for 85 min.

Table 3: primers used in PCR for detection of virulence genes in V. cholerae

| Gene Name | DNA Sequences (5'-3') | Product Size (bp) | References |
|-----------|-----------------------|-------------------|------------|
| tcp       | F CGTTGGCGGTGTCAGTCTTG | 805               | [19]       |
|           | R CGGGCTTCTTCTTGCG    |                   |            |
| toxR      | F CCTTCGATCCCCTAAGCAATAC | 779               | [20]       |
|           | R AGGGTTAGCAAACGATGCAGTAG |           |            |
| ctxB      | F GCCGGGTTGGGAATGCTCCAAG | 536               | [19]       |
|           | R CATGCGATTGCCGCAATTATGACC |          |            |

Table 4: PCR condition of virulence genes in V. cholera

| St. No. | Step            | Temperature ºC | Time   | Number of Cycles |
|---------|-----------------|----------------|--------|-----------------|
| I       | Initial Denaturation | 95             | 5 min  | 1               |
|         | Denaturation      | 94             | 40s    |                 |
| II      | II Annealing      | 59             | 60s    | 30              |
|         | III Extension     | 72             | 90s    |                 |
| III     | Final Extension   | 72             | 10 min | 1               |

2.6 Antimicrobial susceptibility testing (AST) by disk diffusion

All V. cholerae isolates in this study were tested for resistance to six antimicrobials by a disk agar diffusion method [21]. The following antimicrobials were used listed in table (5) and the isolates were characterized as susceptible, intermediate or resistant according to the criteria published by the Clinical and Laboratory Standards Institute (CLSI).

Table 5: Antibiotics discs used in this study, their symbol and concentration.

| No. | Antibiotic | Symbol | Concentration μg. |
|-----|------------|--------|-------------------|
3 Results

The results of isolation for V. cholerae from 300 fecal samples of diarrheic patients appeared that (20) isolates identified (by biochemical test, API-20 system and molecular method by 16s rRNA gene as in figure (1) as V. cholerae. The important clinical signs have been appeared in children were the vomiting with fever; In addition to watery diarrhea.

| No. | Antibiotic                          | Abbreviation | Concentration |
|-----|-------------------------------------|--------------|---------------|
| 1   | Ampicillin                          | AMP          | 10            |
| 2   | Tetracycline                        | TE           | 30            |
| 3   | Ciprofloxacin                       | CIP          | 5             |
| 4   | Chloramphenicol                     | C            | 30            |
| 5   | Nalidixic acid                      | NA           | 30            |
| 6   | Trimethoprim /sulphamethoxazole     | STX          | 25            |

3.1 PCR detection of virulence genes

All V. cholerae isolates were screened for three virulence genes (tcp, toxR and ctxB) by a multiplex PCR method, 65% of isolates were positive for tox R gene, 35% of isolates were positive for tcp gene, while 15% of isolates were positive for ctx B gene as in figure (2) and table (6).

Figure 1: Agarose gel electrophoresis of 16s rRNA gene PCR products amplified from V. cholerae M: marker (2 kb ladder); 1 to 20= lanes: V. cholerae isolates and C is negative control.

Figure 2: Multiplex PCR for analysis of (tcp, toxR and ctxB) genes. Lane (1-13) V. cholerae isolates; lane M: 2000 bp ladder
Table 6: distribution virulence genes in V. cholerae isolates.

| Virulence gene | Number of V. cholerae | Percentage of V. cholerae % |
|----------------|-----------------------|-----------------------------|
| tcp            | 7                     | 15                          |
| toxR           | 13                    | 65                          |
| ctxB           | 3                     | 35                          |

3.3 Antimicrobial susceptibility test
AST revealed high levels of resistance to ampicillin (100%) of V. cholerae isolates, nalidixic acid (90%), sulfamethoxazole-trimethoprim (80%) of isolates, tetracycline and ciprofloxacin (55%) of isolates and chloramphenicol (45%) of isolates as in table (7) and figure (3).

Table 7: Antimicrobial susceptibility testing

| Antimicrobial Agents | V. cholerae isolates | % of R | % of S |
|---------------------|----------------------|--------|--------|
| Ampicillin          | 100%                 | 0%     |        |
| Nalidixic acid      | 90%                  | 10%    |        |
| sulfamethoxazole-trimethoprim | 80% | 20% | |
| Ciprofloxacin       | 55%                  | 45%    |        |
| tetracycline        | 55%                  | 45%    |        |
| Chloramphenicol     | 45%                  | 55%    |        |

(R) Resistance, (S) sensitive

Figure 3: Distribution of antimicrobial resistance between V. cholerae isolates.

4 Discussion
Vibrio cholerae the causative agent of cholera is a major pathogen worldwide, especially in the development countries [22]. Our findings showed that (6.66%) of watery diarrheal patients infected cholera; The socio-cultural and environmental circumstances including poor sanitation, massive religious gatherings and some natural disasters such as severe flooding, facilitates the spreading of the cholera disease. This finding is similar to that reported in Baghdad 2007 cholera outbreak and in Babylon in 2012 and 2014 [23] [24] [25]; while Jasim, (2005) reported only (1.5%) of V. cholerae isolated from watery diarrheal patients in Thi-Qar province these difference may be because the period of study. Since then, several studies have given reports on the prevalence of V. cholerae variants in several Asian countries [26][27]. This cholera genotype did not exist prior to this
period. These results give indication that Iraq cholera disease represents epidemics spreading from some neighboring countries visitors to holy shrine sites in Iraq [28]. This may be due to inadequate supplies and a shortage of health personnel, among other factors which provide adequate conditions for V. cholerae to survive better in the warm environment and crowded areas which give better transmission from human to human by food and water since [29] [30]. These samples were collected according to the cholera surveillance system of WHO. These isolates were diagnosis by conventional biochemical test, AP20 E system and molecular methods by using 16SrRNA. The results appeared all 20 isolates were positive for 16srRNA. Some Vibrio spp. can cause problems owing to variability in biochemical characteristics within species [31] and can become a “viable but non-culturable” (VBNC) organism resulting in unsuccessful isolation of some Vibrio spp. [32][33]. A molecular biological method, such as polymerase chain reaction (PCR). PCR is more sensitive, specific and rapid method for detection of low microbial concentrations and detection of VBNC pathogens than other standard culturing methods f [34][35]. Our study showed that V. cholerae isolates have many virulence genes such as tox R, tcp and ctx B ; Multiplex PCR analysis revealed that 65% of isolates were positive for tox R gene, 35% of isolates were positive for tcp gene, while 15% of isolates were positive for tox R. The presented findings agreement with result published in 2007 [36] which revealed that all strains tested in multiplex PCR analysis showed positive for tcp and toxR virulence genes. On the other hand, 15% of isolates were positive for ctx B gene. Cholera pathogenesis is a complex process and involves synergism of several genes, although cholera toxin (CT) is supposed to be the most important epidemic marker among various toxins produced by V. cholerae [37]. This study reported V. cholerae isolates become resistant to commonly used antibiotics and multidrug resistance has been on a rise. We also found different patterns of antimicrobial susceptibility for V. cholerae isolates obtained from Thi-Qar province and other province and other countries patients with cholera. The results in our study showed high levels of resistance to ampicillin (100%), nalidixic acid (90%), sulfamethoxazole-trimethoprim (80%), tetracycline and ciprofloxacin (55%) and chloramphenicol (45%),where Al-Hashemy (2014) in Baghdad reported the antibiotics susceptibility pattern of V. cholerae were three isolates were sensitive to tetracycline, chloramphenicol and Nalidixic acid , while one isolate was resistance for ampicillin while Kitaoaka et al. (2011) which their results reflect (100%) resistant to nalidixic acid. The results matched with the study of Al-Khafajie (2007) and Al-Naddawii (2010) who indicated the isolate of V. cholerae showed high sensitivity to tetracycline and in Chapek (2000) found that 75% of isolates had tetracycline resistant. From the method for treatment of cholera with tetracycline and water replacement it is surprising and our findings were consistent with the results from studies that have documented an increase in emergence of multidrug-resistant V. cholerae isolates during the outbreaks occurred in the past decade in Thi-Qar province. In some province in Iran during 2008-2010 the comparing the results from study on antimicrobial susceptibility patterns of V. cholerae isolates and the results of this study, showed the same pattern of antimicrobial resistance for sulfamethoxazole-trimethoprim in addition to no resistance to ciprofloxacin. The current study shows that cholera isolates are highly sensitive to ampicillin, gentamycin, cephalothin, cefotaxime + clavulanic acid, amikacin and chloramphenicol (99% - 100%), however, they are less sensitive to tetracycline (37.6%) and erythromycin (12.4%) and highly resistant to nalidixic acid (100%). It is interesting to find that the results of this study were close to the results of other studies reported by several researchers [38] [39] [40] [41]. Yet other researchers reported that V. cholerae is sensitive to nalidixic acid (69.4%), trimethoprim (68.8%), ampicillin (49.4%), erythromycin (38.7%), gentamycin (31.2%) and tetracycline (5%) [42]. The variations in the results may be due to the difference of age, race, ethnicity, travel, and species [13]. Clearly V. cholerae was found resistant to commonly used antibiotics such as tetracycline, erythromycin and sensitive to ceftriaxone, cefixim, such behavior may threat through a higher secondary infection rate and by causing illness of longer duration [42]. In general, it was found that the resistance genes are located on large conjugative elements (SXT constins) that are integrated into prfC on the V. cholerae chromosome [43], and this finding have opened space for more investigations and discussion for studying the integrating conjugative elements as well as the mobile genetic elements that can potentially mediate transfer of antimicrobial drug resistance [44]. Antimicrobial drug resistance in Vibrio spp. can developed by efflux pumps, spontaneous mutation in chromosome, presence of the conjugative plasmids, and trimethoprim/sulfamethoxazole (SXT) elements and integrons [45]. In addition to the factors related to the microorganism itself and horizontal gene transfer ;Antimicrobial resistance patterns in V. cholerae strains can different greatly in depending on geographical location, patterns of antibiotic consumption among the studied people, and the period of study, the lacking or inadequate regulation of antibiotic consumption, inappropriate implementation of existing laws, and population movements are among people and the behavioral factors that related to the emergence of multidrug-resistant microorganisms (MDR) in development countries [46].
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