

The Isolation of *Vibrio cholera* and Other Enteric Bacteria with Molecular Characterization of *Vibrio cholera* during the Outbreak of Baghdad/Iraq in 2015

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Received 19 June 2016; accepted 8 August 2016; published 11 August 2016

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Abstract

Vibrio cholera, causing acute watery diarrhea known as cholera disease, affects all ages and both genders. Cholera infection outbreaks in Iraq have been reported for several years. The recent cholera outbreak, emerged throughout 2015, was investigated using bacteriological laboratory tests, singleplex and multiplex PCR technique for the detection of *V. cholera* from stool samples. Furthermore the toxigenic potential coupled with the antibiotic susceptibility test for cholera and other bacteria were also investigated. The stool samples were collected from 5698 patients admitted to Al-Yarmouk Teaching hospital and health care centers in Baghdad/Al-Karkh, Iraq, from the 1st of August to the 30th of December 2015. The *V. cholera* was isolated from 194 cases (3.4% of the cases age between 21 - 50 years). In addition, other enteric infections: Salmonellosis and Shigellosis 7 and 21 respectively, protozoan parasite *Giardia lamblia* and *Entamoeba histolytica* 2 and 43 cases respectively were also reported. High percentage of *V. cholera* infection was detected in October (122 cases, 62.8%), compared with other enteric infections that show high percentage of diarrheal disease in September and November. The results have confirmed that the cholera outbreak was caused by *V. cholera* O1, biotype El Tor, and serotype Inaba. Seven virulence genes were identified *ctxA*, *toxR*, *zot*, *ace*, *rfbO1*, *tcpA* and *ompW*. Moreover, the cholera isolated strains were found sensitive to most antibiotic but resistant to nalidixic acid.

Keywords

V. cholera, Enteric Bacteria, Diarrhea Outbreak, Virulence Factor Genes, Molecular Identification,

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How to cite this paper: Jameel, S.K., Shafek, M.A., Abdulmohsen, A.M., Mohamed, N.S., Naji, S.R. and Mohammed, T.T. (2016) The Isolation of *Vibrio cholera* and Other Enteric Bacteria with Molecular Characterization of *Vibrio cholera* during the Outbreak of Baghdad/Iraq in 2015. *Advances in Microbiology*, 6, 699-715. <http://dx.doi.org/10.4236/aim.2016.69069>

Antimicrobial Sensitivity Test

1. Introduction

Across the globe, infectious gastroenteritis (IGE) is a cause of global morbidity and mortality [1]. There are different etiologic agents that may cause IGE, including bacteria (*Shigella*, *Campylobacter jejuni*, *Vibrio cholera* O1 and 139 and other pathogenic Vibrios), viruses (*rotavirus*) and parasites ((protozoa: *Giardia lamblia* and *Entamoeba histolytica*) and helminthes) [2] [3]. Diarrhea is the dominant symptom of IGE, it represent more than 30% of hospitalizations morbidity [4], usually begins as acute watery or as dysentery bloody [5] [6], then leads to weight loss and may cause dehydration [7]. It's common in all age groups especially in infants and young children, because their digestive and immune systems are still developing and vulnerable to infection [8]. Enteric infections commonly transmitted through fecal-oral route (food and water contamination) [9]. To reduce this risk of the transmission, a good personal hygiene practice and good food handling practice are required [10]. However, it has been suggested that people who have enteric infections should be excluded from the work in a high risk setting (*i.e.* workers in health care, residential care and child care, food handlers, young children in child care, etc.) because those people represent a source of IGE even when they are clinically asymptomatic for 24 - 48 hrs and have normal stools test [11].

Vibrios are Gram-negative, highly motile curved rods with a single polar flagellum. They tolerate alkaline media that kill most intestinal commensal, but they are sensitive to acid. Numerous free-living vibrios are known, some potentially pathogenic. Until 1992, cholera was caused by only two serotypes, Inaba (AC) and Ogawa (AB), and two biotypes, classical and El Tor, of toxigenic O group 1 *V. cholera* [12]-[14]. The bacterium may be identified by agglutination in O group 1-specific antiserum directed against the lipopolysaccharide component of the cell wall and by the demonstration of their enterotoxigenicity. In 1992, cholera caused by serogroup O139 (synonym "Bengal" the 139 and latest serogroup of *V. cholera* to be identified) emerged in epidemic proportions in India and Bangladesh. This serovar is identified by 1) absence of agglutination in O group 1 specific antiserum; 2) by agglutination in O group 139 specific antiserum; and 3) by the presence of a capsule [12].

The pathogenesis of *V. cholera* involves infection, reaching and colonizing in the epithelium of the small intestine. Within the intestine, where *V. cholera* express a variety of extracellular aggressive virulence factors, toxin coregulated pilus (TCP) and cholera toxin (CT) are essential for the colonization of the host and enterotoxicity, respectively [15] [16]. The virulence cascade *toxR* protein of *V. cholera* regulates the expression of several virulence factors including TCP that play important roles in the pathogenesis of cholera [17]. There are at least 6 virulence genes in *V. cholera*. These genes are the regulator factors and they are: *toxR*, cholera toxin (*ctxA*), toxin coregulated pilus (*tcpA*), outer membrane protein (*OmpW*), accessory cholera toxin (*ace*), zonulaoccludens toxin (*zot*) and O1 somatic antigen (*rfbO1*) which may commonly be found in all *V. cholera* species [18] [19]. People infected with cholera may suffer from different clinical symptoms including vomiting, fever, hypotension, removes essential body fluids, salts and vital nutrients. They may also suffer from body spasm, paralysis and fainting [20] [21]. Patients with severe dehydration, as a result of diarrhea caused by *V. cholera*, are recognized and they are either treated with oral rehydration fluid and/or intravenous fluid replacement, while antibiotics are not a necessary part of cholera treatment, because it may contributes to the emergence of resistance [22] [23]. Other researchers confirmed to use different antibiotics like trimethoprim-sulfamethoxazole, tetracycline, azithromycin, erythromycin, and ampicillin, which are very useful to reduce the duration of cholera diarrhea [15] [24].

To assist with confirmation of *V. cholera*; numerous diagnostic laboratory tests are applied [25] [26] including stool culture, biochemical test, specialized microscopy, serological test and these include polyvalent, Ogawa, Inaba antisera testing, and singleplex PCR, conventional coupled PCR or real-time PCR with fluorescence; for detecting different genes encoding to the different *V. cholera* toxins [18] [27].

Cholera Outbreak in Iraq 2015

In the last two decades, the cholera disease in Iraq is represented as an epidemics infection [28]. This has worsened

especially after 1991 the first Gulf war when the cholera became endemic in all governorates of Iraq [29]. Between 2007 and 2009, many Iraqi people suffered from the cholera disease [24]. At that time, *V. cholera* O1 serotype (Inaba) was detected and confirmed for. In 2012, *V. cholera* O1 serotype (Ogawa) was detected and confirmed for [14] [30]. The climatic factors in Iraq may play a role in *V. cholera* outbreaks and also in the strength, duration, or appearance of these annual seasonal patterns, especially in countries near the equator which have higher and more constant temperatures and thus may encourage the constant level of cholera outbreaks mostly in the warmer months [31]. Furthermore, most of wastewater treatment in Iraq has not been functioned according to golden roles due to the war. This has caused a huge contamination with *V. cholera* in freshwater across the country. Between September 23 to November 6, 2015, 2651 cholera cases have been reported and confirmed in Baghdad and in other 14 Iraqi governorates; such as serogroup O1 (Inaba serotype) with a few cases of (Ogawa serotype) which affected different age groups and genders [32]. Accordingly, the WHO recommended the necessity to improve water supplies and the infrastructures as well as to equip electricity and sanitary disposal of feces in addition to the betterment of the physical and social conditions especially in the refugee camps, for the control of *V. cholera* outbreaks [33] [34].

The aim of this study is the isolation and identification of the enteric pathogen, the *V. cholera* in particular, to determine the main source of the diarrhea with the toxigenic (virulent genes) local isolates of *Vibrio cholera* from the patients admitted to Al-Yarmouk Teaching hospital and from Medical Sectors (health care centers) and identify the antibiotic susceptibility for *Vibrio cholera* and other enteric bacterial isolates.

2. Materials and Methods

2.1. Collection of Stool Specimens

Suspected cholera stool specimens were collected from patients at Al-Yarmouk Teaching hospital and 38 health care centers, Baghdad/Al-Karkh. In this surveillance system, every patient with diarrhea attending the hospital or the care centers screened for major enteric pathogens. According to the hospital surveillance records, 82% of the patients report to Al-Yarmouk Teaching hospital and 38 health care centers within 1 day after the first episode of acute diarrhea, while 18% have report between 1 and 2 days. For this study, water stool samples were collected aseptically from 5698 patients who had no prior medication, from 1st of August to 30th of December 2015 and the participants are males and females.

The stool samples that are collected without transport media were immediately transported to the laboratory within 30 min; while from health care center, Cary-Blair transport medium was used. All samples (except stool swab) were examined microscopically for the presence of red and white blood cells, yeast and parasites to assess colonic inflammatory processes. However, to detect ova and parasites trophozoites and/or cyst like *Giardia lamblia*, and *Entamoeba histolytica*, the Lugol iodine solution was used [2] [35].

2.2. Bacterial Culture, Isolation and Identification

Collected stool specimens were immediately placed into alkaline peptone water (APW; Biomark™, India) at pH 8.5 as an enrichment media for vibrios [36], and incubated at 37°C for 6 hrs., then streaked using an inoculating loop onto selective media thiosulfate citrate bile salts sucrose (TCBS), MacConkey agar, and blood agar (Biomark™, India) and incubated at 37°C for 18 to 24 h [37].

Regarding the isolation of *Salmonella*, the stool samples were inoculated into selective enrichment media tetrathionate broth with Iodine-iodide solution (Ibn Al-betar Research Center, Iraq) and incubated at 37°C for 18 to 24 hrs., then streaked using an inoculating loop onto selective media, Xylose lysine deoxycholate agar (XLD; Conda, Spain) and Hektoen agar (HK; Biorex, UK) then incubated at 37°C for 18 to 24 hrs. For *Shigella*, stool sample have been inoculated directly into the selective media [38] [39].

2.3. Biochemical Tests

All bacterial isolates *Vibrio*, *Salmonella* and *Shigella* were diagnosed according to their biochemical tests the Oxidase, Indole, kligler Iron agar (KIg)-slant-(sugar ferment-gas-H₂S), Urease, Simmon citrate media, API 20 E and API 20 NE (Biomereumix, France). In addition to Cholera Red, String test and Polymyxin B disc were done for Vibrios [15]. All bacterial isolates were sent to the Central Public Health Laboratory (CPHL), Ministry of Health/Baghdad-Iraq to prove it *Vibrio cholera*, *Salmonella* and *Shigella*.

2.4. Serogrouping

V. cholera colonies from KIg; (Biorex, UK) slant were tested to identify their serogroups using slide agglutination with polyvalent anti-Ogawa and anti-Inaba antisera (Denka seiken co. LTP-JAPAN) [40]. *V. cholera* O1 were preserved at -20°C in glycerol for subsequent detection of *V. cholera* by multiplex PCR.

2.5. DNA Preparation

DNA extraction was carried out according to the enrichment by culturing in alkaline peptone water (1% Bacto Peptone, 0.5% NaCl, 0.07% Na_2CO_3 , 0.01% KNO_2 , pH 8.6) for 24 hrs. at 37°C . Grown cultures were plated on TCBS agar plates and Kliglar iron slant. All isolates were routinely grown on Luria-Bertani (LB; Oxoid, UK) agar throughout the study. The Wizard® Genomic DNA Purification Kit (Promega, USA) was used according to the manufacturer's instructions. The *V. cholera* DNA detection were done using agarose gel electrophoresis which viewed with the red safe DNA stain and UV transilluminator (BioRad, USA).

2.6. PCR Assays

The PCR reaction was in 40 μl of a 5X FIREPol® Master Mix Ready to Load (solisBioDyne). Optimization of multiplex PCR was accomplished after several trials. Amplification was conducted using the following conditions: 1 cycle pre-denaturation at 95°C for 10 min, 35 cycles of secondary denaturation at 95°C for 1 min, annealing at 61.5°C for 1 min, extension at 72°C for 1.2 min, and final extension at 72°C for 10 min. The amplified products were separated with 2.5% agarose gel and viewed using transilluminator (BioRad, USA).

2.7. Sequencing and Data Analysis

For the genes (*ctxA*, *tcpA*, *ace*, *rfbO1*, *toxR*, *ompW* and *zot*) using the cleanup PCR by (microClean,) using genetic analyzer 3100 (Applied Biosystems, USA) data were analyzed using Sequencing Analysis 5.2. The genes sequences submitted to NCBI/Gene bank database with accession numbers KX096224-KX096236, respectively.

2.8. Antibiotic Susceptibility Profile of Bacterial Isolates

All bacterial isolates were subjected to Kirby-Bauer method for susceptibility test, using disk diffusion technique; it was accomplished on Muller-Hinton agar [41]. For *V. cholera*, the following antibiotics are used: amikacin, ampicillin, cephalothin, cefotaxime, chloramphenicol, gentamicin, erythromycin, nalidixicacid, and tetracycline (Al-Razi Diagnostics Center/Iraq and Bioanalys /Ankara-Turkay). For *Salmoella* and *Shigella* the following antibiotics were used amoxicillin/clavulanic acid, tetracycline, ciprofloxacin, cefotaxime, ceftriaxone, azothromycin, aztreonam, co-trimoxazole, amoxicillin, ampicillin, chloramphenicol, imipenem (Al-Razi Diagnostics Center/Iraq and Bioanalys /Ankara-Turkay) [24] [42]-[44].

2.9. Statistical Analysis

Data were submitted to statistical analysis using EpiInfo-6 software program. Tables of frequency and percentage were issued. Chi-square test was calculated to explore the significance of the relationship between different variables, P value ≤ 0.05 was considered as positive level of significance.

3. Results

3.1. Isolates

From 1st of August to 30th of December 2015, 5698 stool samples were examined. The result was 194 as *V. cholera*, 21 as *Shigella* spp. (mostly *Shig. flexneri*), and 7 *Salmonella* spp. (mostly *Sal. typhi*). All *Vibrio cholera* isolates belong to the pattern of O1 serogroup, biotype-El-Tor, serotype Inaba. Protozoan parasite *Giardia lamblia* 2 and *Entamoeba histolytica* 43 were also reported.

3.2. Infection Pattern and Seasonality

It is clear that **Table 1** shows the significant results ($P < 0.05$) of cholera infection. Two peaks were detected in

different months: the first peak in September (57 cases) and increased in October (122 cases), followed by the gradual decline in November (14 cases) with only one case in August, finally, in December, no positive case has been recorded. **Table 2** reveals that there is no-significant result ($P > 0.05$) of cholera infection and other enteric pathogen between the two genders. From 267 of different pathogens present in the study, **Table 3** clearly shows that the highest percentage of *V. cholera* infection has occurred during October (122 cases (62.9%) compared with the highest percentage of *E. histolytica* (28 cases (65.1%) and *Shigella* infection (7 cases (33.3%) in November. Regarding *Salmonella*, there were only 3 cases reported (42.8%) during September. Concerning *G. lamblia*, the results revealed only 2 cases distributed in November and December.

The results in **Table 4** demonstrate that the cholera infections are distributed for all age groups by both gender ($P > 0.05$). Moreover, the highest percentage of cholera infections was found in the age group of 21 - 30 y (56

Table 1. Distribution of positive cholera cases according to month of occurrence and gender.

Variable	Positive <i>V. cholera</i> No. (%)	Negative <i>V. cholera</i> No. (%)	Total No. (%)	P-value*
Month**	August	1 (0.3)	292 (99.7)	0.000001 df = 3
	September	57 (4.5)	1207 (95.5)	
	October	122 (4.5)	2575 (95.5)	
	November	14 (1.4)	960 (98.6)	
	December**	-	470 (100)	
	Total	194 (3.4)	5504 (96.6)	
Gender	Male	107 (3.7)	2785 (96.3)	0.212 df = 1
	Female	87 (3.1)	2719 (96.9)	
	total	194 (3.4)	5504 (96.6)	

**December doesn't included in the statistical analysis because there is no cholera case.

Table 2. Presence of different pathogens in the study stool sample by gender.

Pathogen	Male No. (%)	Female No. (%)	Total No. (%)
<i>V. cholera</i>	107 (55.2)	87 (44.8)	194 (100)
<i>Salmonella</i>	2 (28.6)	5 (71.4)	7 (100)
<i>Shigella</i>	10 (47.6)	11 (52.4)	21 (100)
<i>Entamoeba histolytica</i>	22 (51.2)	21 (48.8)	43 (100)
<i>Giardia lamblia</i>	1 (50)	1 (50)	2 (100)
Total	142 (53.2)	125 (46.8)	267 (100)

Table 3. Presence of different pathogens in the study stool sample according to month.

Months	No.	<i>V. cholera</i>	<i>Sal.</i>	<i>Shi.</i>	<i>E.H.</i>	<i>G.L.</i>	Total
August	293	1 (0.5)	2 (28.6)	4 (19.0)	-	-	7
September	1264	57 (29.4)	3 (42.8)	5 (23.8)	1 (2.3)	-	66
October	2697	122 (62.9)	2 (28.6)	4 (19.0)	5 (11.6)	-	133
November	974	14 (7.2)	-	7 (33.3)	28 (65.1)	1 (50)	50
December	470	-	-	1 (4.8)	9 (20.9)	1 (50)	11
Total	5698	194 (100)	7 (100)	21 (100)	43 (100)	2 (100)	267

Sal. = *Salmonella*, *Shi.* = *Shigella*, *E.H.* = *Entamoeba histolytica*, *G.L.* = *Giardia lamblia*.

cases; 28.9%), followed by the age groups of 31 - 40 y (42 cases; 21.7%), and 41 - 50 y (35 cases; 18.0%), while less cholera infections have occurred in the age groups below 10 and over 60 y, 13.7 and 13 cases (3.6% and 6.7%) respectively. In general, for the age groups between 11 - 20 y and 51 - 60 y, the cholera infection has affected all of them equally.

3.3. The Distribution of Patients with Cholera According to the Different Sources (Sector/District, Medical Center and Hospital)

The distribution of cholera infection by gender and sectors/districts is illustrated in **Table 5**. The highest percentages were from Al-Alam sector 74 cases (38.1%) followed by Al-Amil sector 54 cases (27.8%) then Al-Dora sector 38 cases (19.6%), while Al-Karkh and Al-Adel sectors, recorded the convergent results 7.7% and 6.7%, respectively. Furthermore, there is no-significant difference between the cholera infection for gender and the residence in different districts ($P > 0.05$). Nevertheless, statistically high significant value ($P = 0.0001$) between 154 positive cases infected with *V. cholera* (9.9%) were admitted to Al-Yarmouk Teaching hospital, compared with 40 positive cases infected with *V. cholera* (0.96%) from other medical health care centers (**Table 6**).

Table 4. Distribution of cholera cases according to age groups and gender.

Age/years	Positive <i>V. cholera</i>		Total positive	P-value
	Male (+ve)	Female (+ve)		
-10	1	6	7	
11 - 20	15	5	20	
21 - 30	32	24	56	
31 - 40	24	18	42	0.273 <i>df</i> = 5
41 - 50	13	22	35	
51 - 60	13	8	21	
61-	9	4	13	
Total	107	87	194	

Table 5. Distribution the positive cases of *Vibrio cholerae* by gender and sectors.

Sector/district	Positive <i>V. cholera</i>		Total Positive	P-value*
	Male	Female		
AL-Karkh	12 (80)	3 (20)	15 (7.7)	0.35 <i>df</i> = 4
AL-Adeel	8 (61.5)	5 (38.5)	13 (6.7)	
AL-Amil	28 (51.9)	26 (48.1)	54 (27.8)	
AL-Alam	40 (54.1)	34 (45.9)	74 (38.1)	
AL-Dora	19 (50)	19 (50)	38 (19.6)	
Total	107 (55.2)	87 (44.8)	194 (100)	

Table 6. Distribution of examination results according to source of the sample.

Source of sample	Positive for <i>V. cholera</i>	Negative for <i>V. cholera</i>	Total	P-value*
Medical health care centers	40 (0.96)	4109 (99.04)	4149	0.0001 <i>df</i> = 1
AL-Yarmouk Teaching Hospital	154 (9.9)	1395 (90.01)	1549	
Total	194 (3.4)	5504 (96.6)	5698	

3.4. Primer Design

The primer sequence was designed for *ompW* and *zot* genes in-silico using primer 3 algorithm and, as a result, good primers have appeared for the detection of these genes (Table 7). The primers for *ctxA*, *ace*, *toxR*, *rfbO1* and *tcpA* genes were designed according to Fields, Goal and Rivera references [45]-[47].

3.5. Genotyping by PCR

The isolates 194 *V. cholerae*, in which only 35 isolates were confirmed genetically, have the *ompW* specific species gene (Figure 1). The detection of the toxigenic strain of *V. cholera* that possessed the CTX genetic element

Table 7. Primer sets used for PCR assay and the amplicons size.

NO	Primer name		Primer sequence	Amplicons size	Reference
1	<i>ompW</i>	F	5'-TGCCTCGGTAGTACCTAATGA-3'	486 bp	This study
		R	5'-GCACCAGCTTTGTAGGTTGC-3'		
2	<i>tcpA</i>	F	5'-CGTTGGCGGTCAGTCTTG-3'	805 bp	Goel et al. 2007
		R	5'-CGGGCTTTCTTCTTGTTTCG-3'		
3	<i>rfbO1</i>	F	5'-TCTATGTGCTGCGATTGGTG-3'	538 bp	Goel et al. 2007
		R	5'-CCCCGAAAACCTAATGTGG-3'		
4	<i>toxR</i>	F	5'-CCTTCGATCCCCTAAGCAATAC-3'	779 bp	Rivera et al. 2001
		R	5'-AGGGTTAGCAACGATGCGTAAG-3'		
5	<i>ctxA</i>	F	5'-CGGGCAGATTCTAGACCTCCTG-3'	564 bp	Fields et al.1992
		R	5'-CGATGATCTTGGAGCATTCCCAC-3'		
6	<i>ace</i>	F	5'-TTGATGGCTTACGTGGCTT-3'	198 bp	Rivera et al. 2001
		R	5'-GCGCTTGGTCTAACCCTAAA-3'		
7	<i>zot</i>	F	5'-GGTGGCTTTGACATGCATC-3'	575 bp	This study
		R	5'-CCAAATTGTCTACGAGGCCGA-3'		

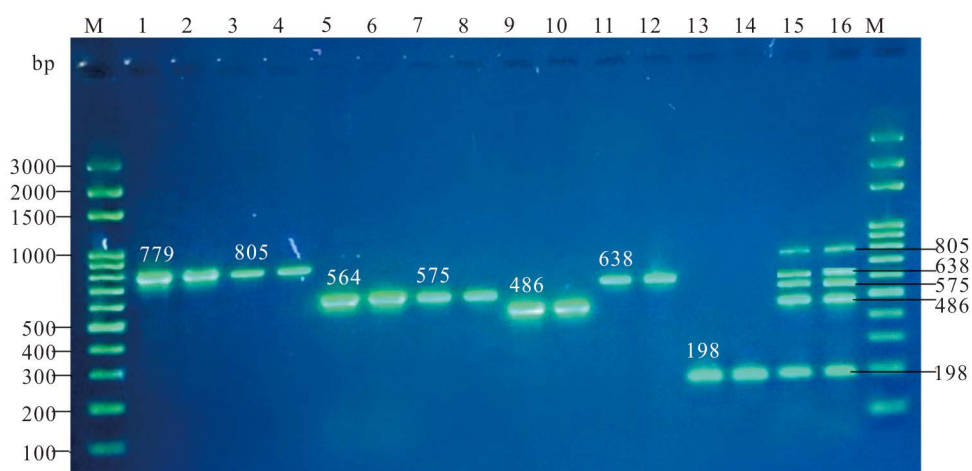


Figure 1. Agarose gel electrophoresis result of uniplex PCR products. Lane M: 100 bp ladder, lanes 1, 2 represent *toxR* gene PCR products (779 bp), lane 3, 4 represent *tcpA* gene PCR products (805 bp), lane 5, 6 represent *ctxA* gene PCR products (564 bp), lane 7, 8 represent *zot* gene PCR products (575 bp), lane 9, 10 represent *ompW* gene PCR products (486 bp), lanes 11, 12 represent *rfbO1* gene PCR products (638 bp), lane 13, 14 *ace* gene PCR products (198 bp), lanes 15, 16 represent multiplex of five gene (*tcp*, *rfbO1*, *zot*, *ompW*, *ace* respectively). Electrophoresis was carried out in 2.5% agarose gel supplied with Red safe DNA dye at (5 V/cm) for 2 hrs.

contains *ctxA* gene, *toxR*, *zot*, *ace*, *rfbO1*, *tcp* and *ompW* genes using singleplex PCR for each single gene were tested and optimized for several trials till reached to the best reaction condition (**Figure 1**). Moreover, PCR analysis (Singleplex and Multiplex) revealed that all randomly 35 isolates tested are positive for *ctxA*, *toxR*, *ace*, *rfbO1*, *tcpA* and *ompW* genes (100%). In comparison, only 31 isolates (88.57%) are positive for *zot* gene. Noteworthy, the *ompW* genes is a marker for the identification of *V. cholera* which was shown to be dominant in all of the investigated isolates.

3.6. Antimicrobial Drug Susceptibility

Disk diffusion testing demonstrated that all isolates of *V. cholera* O1 were highly sensitive (100%) to ampicillin, cefotaxime + clavulanic acid and cephalothin, followed by chloramphenicol, gentamycin and amikacin (99%) (**Table 8**). Nevertheless, *cholera* isolates show less sensitivity to tetracycline and erythromycin with the rate sensitivity of 37.6% and 12.4%, respectively, whereas all were resistant to nalidixic acid (100%).

The 21 isolates of *Shigella* and 7 isolates of *Salmonella* were sensitive (100%) to ciprofloxacin, aztreonam, ampicillin, chloramphenicol and imipenem. Moreover, *Salmonella* alone is (100%) sensitive to tetracycline, ceftriaxone, co-trimoxazole, but it shows less resistance to amoxicillin, amoxicillin + clavulanic acid (28.6%, 14.3%) respectively, in comparison with *Shigella* which shows high resistance to these two types of antibiotics (85.7%, 76.2%) respectively. In general both types of bacteria demonstrate acceptable degree of sensitivity test to cefotaxime and azithromycin (**Table 9**).

4. Discussion

Phenotypic results, obtained in this study, led to presumptive identification of the Baghdad/Al-Karkh 194 bacterial isolates as the *V. cholera* O1 serogroup, biotype El Tor and serotype Inaba. This finding is similar to that reported in Baghdad 2007 cholera outbreak and in Babylon in 2012 and 2014 [28] [37] [55]. This new variant of *V. cholera* was first reported in Bangladesh 2002 [48]. Since then, several studies have given reports on the prevalence of *V. cholera* variants in several Asian countries [49]-[54]. 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. cholera* O1, biotype El Tor, serotype Inaba to survive better in the warm environment and crowded areas which give better transmission from human to human by food and water since the El Tor strains have efficiency transmission from host to host when compared with classical cholera strains [56] [57].

Table 8. Sensitivity of cholera isolates to the antibiotics *in vitro*.

Antibiotic disc	<i>V. cholera</i> isolates (N = 194)		
	Sensitive	Moderate	Resistant
	No. (%)	No. (%)	No. (%)
Amikacin	192 (99)	1 (0.5)	1 (0.5)
Ampicillin	194 (100)	-	-
Chloramphenicol	193 (99.5)	-	1 (0.5)
Cefotaxime + Clavulanic acid	194 (100)	-	-
Cephalothin	194 (100)	-	-
Erythromycin	24 (12.4)	170 (87.6)	-
Gentamycin	193 (99.5)	1 (0.5)	-
Nalidixic acid	-	-	194 (100)
Tetracyclin	73 (37.6)	121 (62.4)	-

S = sensitive, M = moderate sensitivity, R = Resistant.

Table 9. The sensitivity test of *Salmonella* & *Shigella* isolates to the antibiotics discs.

Antibiotic disc	<i>Shigella</i> sp (N = 21)			<i>Salmonella</i> sp (N = 7)		
	S	M	R	S	M	R
Amoxicillin + Clavulanic acid	5 (23.8)	-	16 (76.2)	5 (71.4)	1 (14.3)	1 (14.3)
Tetracyclin	18 (85.7)	-	3 (14.3)	7 (100)	-	-
Ciprofloxacin	21 (100)	-	-	7 (100)	-	-
Cefotaxime	13 (61.9)	-	8 (38.1)	5 (71.4)	-	2 (28.6)
Ceftriaxone	20 (95.2)	1 (4.8)	-	7 (100)	-	-
Azithromycin	19 (90.5)	-	2 (9.5)	6 (85.7)	1 (14.3)	-
Aztreonam	21 (100)	-	-	7 (100)	-	-
Co-Trimoxazole	5 (23.8)	1 (4.8)	15 (71.4)	7 (100)	-	-
Amoxicillin	3 (14.3)	-	18 (85.7)	3 (42.8)	2 (28.6)	2 (28.6)
Ampicillin	21 (100)	-	-	7 (100)	-	-
Chloramphenicol	21 (100)	-	-	7 (100)	-	-
Imipenem	21 (100)	-	-	7 (100)	-	-

S = sensitive, M = moderate sensitivity, R = Resistant.

In this context, it is noteworthy that the few studies suggested that most of the Iraqi cholera isolates mostly belong to Ogawa serotype and only few are Inaba serotype [32] [58]. Nevertheless, other studies conducted in Iraq revealed that 58 isolates out of 66 *V. cholera* belong to the pattern under serum Inaba and only 5 isolates are ogawa and 3 isolates belonging to non-cholera vibrios [59]. However, our results are not in agreement with a study done in Baghdad during the 1999 outbreak of cholera [60] and in Haiti from 2010 to 2011 [61] which showed that Ogawa serotype is the predominant. These results may give observation about *V. cholera* serotype which may change epidemiologically when compared with the past decade [37].

In the current study, the rate of diarrhea is approximately similar in male and female. The analysis of this study revealed significant higher occurrence of *V. cholera* infection in September and October than the other months. The present results are consistent with other studies [31] [32]. Other studies described two annuals of El Tor *cholera* peaks; these are a smaller spring outbreak in April before the monsoons rains then a larger outbreak from September to December after the monsoon rains [62] [63]. The primary transmission of *cholera* to the humans have been enabled by several environmental factors such as temperature, salinity, nutrient concentrations, and the plankton in water, shellfish consumption, and aquatic ecosystems [64], due to ecological responses to recent climate change [62], in which current climate condition may enable *V. cholera* to persist in aquatic ecosystems during winter [31].

Other important enteric bacterial pathogens such as *Salmonella* and *Shigella* were also isolated in this study during August to October (*Salmonella* 7 and *Shigella* 21). Furthermore the current study also shows that *E. histolytica* and *G. lamblia* have a higher occurrence during November and December. It is well known that enteric infections, caused by the protozoan and bacteria, exhibit seasonal variation. Enteric bacterial infections rise in the summer and decline in the winter, in contrast to protozoan infection which has shifted towards autumn [65] [66].

The incidence of diarrhea infections caused by enteric bacteria and protozoan occurred around the year and for the whole age groups [8] [34] [67]. They are considered as a waterborne and food-borne infection [66], that increase with the increase of temperature, especially in summer when there is water shortage, higher water consumption, outdoor activities and recreational water use such as camping or swimming, then spread infections person-to-person and may amplify outbreaks of enteric diarrhea disease [68]-[70]. These conditions, after 2003 are clearly visible in many parts in Iraq such as poor environmental sanitation, coupled with special events such as flood, famine, and refugee movements with overcrowded camps which may assist to increase enteric diarrhea

diseases including cholera [71]-[73].

In the present study, 267 (4.7 %) enteric pathogens, including cholera and protozoa, were isolated out of 5698 diarrhea cases, while there have been 5431 (95.3%) negative cases. The interpretation of this result is that the pathogen was present in the stool sample but was below the limit of detection of the assay. Furthermore, stool sample represent a complex matrix with multiple pathogenic microbes, and finding a rare entero-pathogen is inherently challenging [21] [74] [75]. Cholera infection was detected for all age groups and in both gender ($P > 0.05$). The highest percentage was found in adult age groups, while less percentage of cholera infection was found in children and older age groups, and in male (55.2%) than in female (44.8%). These percentages are widely acceptable for the cholera infection outbreak in 2015, which was confirmed that 2651 cases comprising of 1477 (55.7%) males and 1174 (44.3%) females, all age groups were included [32], and, similar to previous researches, it has been shown that age groups were ranged from 12 - 65 years old [76], and 54.4% are male and 45.6% are female out of 160 cases of cholera infections [33]. On the other hand, these results are inconsistent with other results that reported cholera infections in age groups < 1 year (16.3%), 1 - 5 years (62.5%) and > 5 years (21.3%) [28]. Interestingly, the studies in 1999, 2009 and 2014 reported that cholera usually affect the age group between 1 - 5 years and occasionally in the neonatal period 2 - 30 days [37] [58] [77]. It is worth noting that cholera infection in children is less likely to occur presumably due to protection afforded by secretory antibody in mother's milk [78]. Nevertheless it has been speculated that if cholera occurs in less than five years age, then it could be due to poor sanitation and educational measures of this age groups [79]. In contrast, older age groups appear to have less cholera infection and this could be related to their acquired immunity gained from repeated exposure of diseases whether symptomatic or asymptomatic infection [80] [81] and furthermore the recurrent infections of cholera are rare as a result of the role of local immune defense in the intestinal mucosa [78].

Regarding the distribution of patients with cholera in the west of Baghdad, the highest percentage of cholera was isolated from Al-Alam and Al-Amil sectors and less in Al-Dora, Al-Karkh, and Al-Adel sectors. Similar results were also reported for the above mentioned sectors of Baghdad [28] [33]. Cholera transmission is a result of fecal contamination of drinking-water which is closely linked to inadequate environmental management [58]. As a result of that, some sectors in Baghdad suffer from the shortage of municipal drinking water. Therefore, the people living in those sectors dig their own wells without using the adequate methods of water treatment, or they transfer their drinking water via unclean tankers [28]. On other hand, infrastructure destruction as a result of the multiple crises which Iraq has suffered from and events such as floods, famine and wars, displacement of populations to inadequate and overcrowded camps for internally displaced persons or refugees [82], all have participated to spread cholera in Iraq and it has become an epidemic that threatens other neighboring countries since bacterial pathogens can be transmitted via birds, air, foods, and rivers.

In the current study, 154 (9.9%) positive cases for cholera infection were admitted to Al-Yarmouk Teaching hospital; and 40 (0.96%) positive cases for cholera infection from the 38 medical health care centers. These samples were collected according to the cholera surveillance system of WHO. The results affirm that surveillance for cholera is a critical component to be controlled. Moreover, detecting and reporting the bacterial strains is important, especially new strains which have the potential to cause new epidemics. Cholera outbreaks must be detected quickly especially in areas that have recently experienced deterioration in water sanitation due to natural disasters, civil strife, or recently established refugee camps. Accordingly, appropriate and rapid responses should be undertaken, such as preparation of preparedness plans, training of healthcare staff and pre-positioning of supplies [83]-[85].

The PCR analysis revealed that all 35 isolates tested from 194 *V. cholera* isolates were positive for *ace*, *ctxA*, *ompW*, *rfbO1*, *tcpA* and *toxR* genes (100%), while only 31 isolates (88.57%) were positive for *zot* gene. Similar results were also reported in 2013, 2015 and 2016 [86]-[89]. The presented findings agree with result published in 2007 [90] which revealed that all strains tested in multiplex PCR analysis showed positive for *ace*, *ctxB*, *hlyA*, *ompU*, *ompW*, *rfbO1*, *rtx*, *tcpA*, *toxR* and *zot* virulence genes. On the other hand, all the isolated 35 *Vibrio* were positive for the *ompW* gene. The *ompW* gene acts as an internal control for *V. cholera* and could be compatible with the biochemical and serological tests which are used to identify the suspects *Vibrio* isolate [91], which plays an important role in the physiology of these bacteria and has an impact on the rapid adaptation of the bacteria to the environment [92] representing a species-specific for *V. cholera* [86].

In general, the present study shows that all tested *V. cholera* isolates belong to O1 serogroup. The somatic O-antigen biosynthesis gene was positive for O1serogroup (*rfbO1*), in addition to *ctxB*, *zot*, *toxR* and *ompW* genes. Furthermore the *cholera* O1 isolates were positive form accessory *cholera* toxin (*ace*) and toxin

coregulated pilus (tcp). *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. cholera* [91]. Yet the combination of two target genes including *ctxA* and *rfbO1* can be used for differentiation of toxigenic *V. cholerae* O1 from non-toxigenic because *ctxA* gene is mainly responsible for watery diarrhea of toxigenic *V. cholera* [93]. It was found that both clinical and environmental *V. cholera* strains carrying *ctxA*, *tcpA*, *ace* and *zot* genes belonged to El Tor biotype [94]. Therefore, we can conclude that, in the present study, the molecular results are compatible with the bacteriology and serology results which indicated that Baghdad/ Al-Karkh section *cholera* outbreaks in 2015 was caused by a toxigenic *V. cholerae* O1 the El Tor-Inaba. Thus, several studies have shown that the patients who suffered from moderate *cholera* diarrhea, associated with isolated *V. cholera*, lack the *zot* gene which is considered acceptable because of the potential function of this gene due to the increase of the permeability of the mucosa in small intestinal by affecting the structure of the intercellular tight junction [95]. On other hand, the non-enterotoxigenic *V. cholerae* cells that lack *ace*, *zot*, and *ctx* genes do not cause diarrhea in volunteers. Interestingly, the presence of ToxR protein is important for the regulation and function of other virulence genes in pathogenic *V. cholera* [86] [89] [96] [97].

The current study shows that *cholera* isolates are highly sensitive to ampicillin, cefotaxime + clavulanic acid and cephalothin, chloramphenicol, gentamycin and amikacin (99% - 100%), whilst, they are less sensitive to tetracycline (37.6%) and erythromycin (12.4%) and completely 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 [15] [20] [28] [98]. Yet other researchers reported that cholera is sensitive to ampicillin (49.4%), tetracycline (5%), gentamycin (31.2%), trimethoprim (68.8%), erythromycin (38.7%) and nalidixic acid (69.4%) [33] [58] [99] [100]. The variation in the results attributed to the difference of race, ethnicity, age, travel, and species [43]. Clearly *V. cholera* was found resistant to a commonly used antibiotics such as tetracycline, erythromycin and sensitive to ceftriaxone, cefixim; such behavior may threaten through a higher secondary infection rate and by causing illness of longer duration [33]. 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. cholera* chromosome [100], and this finding has 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 [61] [101].

Regarding *Shigella* isolates, they were found to be sensitive to ciprofloxacin, aztreonam, ampicillin, chloramphenicol and imipenem, tetracycline, ceftriaxone, cefotaxime and azithromycin and they are relatively resistant to co-trimoxazole, amoxicillin and amoxicillin + clavulanic acid. Similar results were also reported [9] [43]. Overusing antibiotics to treat shigellosis may contribute to make this bacterium more resistant to the antibiotics [102]-[104]. Moreover, *Salmonella* isolates showed sensitivity to most of the above mentioned antibiotics [42] [105] [106].

5. Conclusion and Recommendation

Cholera isolates were identified as *Vibrio cholera* O1 biotype- El Tor serotype Inaba and these isolates harbored various toxigenic and pathogenic genes (*toxR*, *ctxA*, *tcpA*, *ompW*, *ace*, *zot*, *rfbO1*) and recorded in the NCBI—as an Iraqi cholera isolates outbreaks in 2015. The study revealed that the highest rate of cholera infection was during September to October with the highest percentage rate among adults aged 21 - 50 years. Beside cholera infection, several enteric pathogens were isolated in September to November.

The current study emphasizes the importance to provide a safe drinking-water from a plants and water projects that utilize chlorine for disinfection. Also, it is important to encourage the usage of novel technologies in the disinfection of water. Moreover, there should be strict and accurate liquid waste disposal through competent sewage system to prevent direct throwing of (untreated) waste into the river. Application of the novel processing technique like phages biotechnology (phages-virus) has the potential to improve liquid waste treatment.

Health education plays an important role in the prevention of the disease; better understanding of the disease, identification of mode of transmission, and self-hygiene are the main items in this program.

Furthermore, emphasizing the importance of the adoption of molecular technologies, namely PCR for rapid, qualitative and quantitative cholera diagnosis to replace the microbiological culture media that are time consuming.

Acknowledgements

Grateful thanks to Dr. Ali H. Hayawi for his help in implementing the statistical aspects of this study and to the biologist Aseel K. Hamzah for unlimited cooperation and assistance in the preparation of all culture media required to complete this work.

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