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Importance of *Ompw* gene to diagnose *Vibrio cholerae* isolated from stool and water samples

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ABSTRACT

Background. Cholera is a severe acute watery diarrheal disease caused by O1 and O139 serogroup of *Vibrio cholerae*. The outer-membrane protein W(*OmpW*) a virulence factor, is involved in inducing protective immunity and triggering the immune response. The aim of this study is to find a rapid and accurate method for diagnosing *Vibrio cholerae* and investigate the possibility of *OmpW* gene to achieve this goal, and identify the effective antibiotic against this bacteria.

Methods. The current study included 400 stool samples from patients who were suspected of having cholera and 50 samples of water have been collected from various water liquefaction stations in Thi-Qar province. The samples were examined by routine and advanced biochemical, serological methods and molecular method by using PCR to amplify *16S rDNA* gene and gene *OmpW*.

Results. Results of stool samples were revealed only 41(10.25%) of isolates as *Vibrio cholerae* by biochemical methods while 38 (92.68%) of isolates were diagnosed as Ogawa serotype of *Vibrio cholerae* serogroup O1 by serological and molecular methods. While the water samples did not reveal the appearance of *Vibrio cholerae* by molecular diagnosis. The study revealed there is a difference in the rates of resistance of isolates to some antibiotics, highest percentage rate of resistance (100%) to trimethoprim-sulfamethoxazole while lowest percentage rate of resistance (5.26%) to piperacillin-tazobactam.

Conclusion: All identified isolates were registered in GenBank, two isolates with number LC793854 and LC793858 were recorded as new isolates. *OmpW* gene showed great accuracy to diagnose *Vibrio cholerae*, no less important than the accuracy of *16S rDNA* gene to diagnose of these bacteria, and Piperacillin-tazobactam is the best antibiotic to treatment of cholera.

Keywords: Cholera, *16S rDNA*, *OmpW* gene, piperacillin-tazobactam antibiotic

Introduction

Cholera is a severe lethal secretory diarrheal disease have the potential to become epidemics or pandemics. Every year, four million cases of cholera are reported, accounting for around 140,000 deaths [1]. Severe acute watery diarrheal disease caused by the O1 and O139 serogroup of *Vibrio cholera* [2]. Toxic group O 1 *V. cholerae* has two biotypes, Classical and El Tor, as well as just two serotypes, Inaba and Ogawa, were known to cause cholera [3]. Cholera represents the majority of *Vibrio* infections is affecting individuals of all ages and gender [4]. The most typical

ways for cholera to spread are through eating food that hasn't been cooked properly or contaminated water [5].

The pathophysiology of *V. cholerae* include infection, colonization, and reach-through of the small intestinal epithelium. In the gut, where *V. cholerae* produces several extracellular aggressive virulence factors, toxin coregulated pilus (TCP) and cholera toxin (CT) are required for the colonization of the host and enterotoxicity, respectively[6].The outer-membrane protein W, expressed by the *OmpW* gene and contributes significantly to bacterial pathogenesis by making pathogenic isolates more adaptable. A virulence factor *OmpW* gene, is involved in inducing protective immunity and triggering the immune response [7].

Misdiagnosis of the infection results in serious consequences [8]. Laboratory-based cholera detection methods take time and require well-equipped facilities with professionally trained staff often not available in poor places [9]. However, gene of *16S rDNA* was widely used to identify bacteria and was regarded as the best tool for studying bacterial phylogeny and taxonomy because it is present in all bacteria, its function has not changed over time, and length of *16S rDNA* gene is appropriate [10].

Today, antibiotics resistance is a problem, especially in underdeveloped countries [11]. It rapidly emerged as a result of the extensive use of antibiotics as prophylactic measures during cholera epidemics [12]. Therefore, it is a severe challenge leading to a large proportion of therapeutic failures and resulting in high morbidity and mortality [13]. Piperacillin-tazobactam is one of the antibiotics that hospitalized patients are most usually prescribed. Its broad spectrum of activity against anaerobic, gram-positive, and gram-negative bacteria [14].

Methods

Collection of specimens

A total of 400 stool specimens from patients suspected of having cholera were gathered from all hospitals in province of Thi-Qar, and then transferred via a cold box to the Central Health Laboratory of Thi-Qar using Cary Blair media and 50 samples of water from various water liquefaction stations were examined during the period from September to December 2023.

Isolation of bacteria

All specimens were incubated on alkaline peptone water for six hours at 37 C. After that, they were directly cultured on thiosulfate citrate bile salt sucrose and sub cultured on MacConkey agar and blood agar for 24h at 37 degrees Celsius, and then, a gram stain was performed, biochemical tests were used to diagnose *Vibrio cholerae* [15].

Identification by API 20 E System

Clinically, the analytical profile index is used to quickly identify Enterobacteriaceae, according to (Bio Merieux, France). The tubes' colors change either throughout the incubation period or after the chemicals are applied [16].

Serological tests

Serological slide agglutination was used to identify and distinguish between the *V. cholerae* serogroups and serotypes [17]. Commercial anti-*V. cholerae* O1, anti-*V. cholerae* O139, anti-O1 Inaba and anti-O1 Ogawa antisera were used for this method [18].

Genomic DNA extraction

Genomic DNA of 38 bacterial isolates were extracted by using of Geniod kit. It purification from cultures cultivated, according to the manufacturer's instructions, in brain-heart infusion broth for 18–24 hours. After being separated, the genomic DNA was examined by gel electrophoresis and kept for future use at -20 °C.

Amplification of *16S rDNA* gene

Universal primers of *16S rDNA* gene was amplified by polymerase chain reaction, constituents and program of PCR were shown in table (1 and 2).

Table (1) Constituents of the PCR reaction for *16S rDNA* gene amplifying

Constituents	Volume (µl)
Go taq green master mix	25
DNA template	2
Forward primer	2
Reverse primer	2
Nuclease-Free Water	19
Total volume	50

Table (2) Polymerase chain reaction program for amplifying *16S rDNA* gene

Stages	Temperature	Time	Cycle	Size of product
Initial denaturation	96 °C	5 min	1	1500 bp

Denaturation	96 °C	30 sec	27	
Annealing	56 °C	25 sec		
Extension	72 °C	15 sec		
Final extension	72 °C	10 min	1	
Cooling	4 °C			

Sequencing of PCR products of *16S rDNA*

For purification and sequencing, the amplified *16S rDNA* gene products were sent to the Macrogen firm in South Korea. Gene of *16S rDNA* sequences of 41 bacterial isolates were processed using Bioedit and compared using BLAST tools "http://www.ncbi.nlm.nih.gov" with the NCBI nucleotide sequence databases to determine the sequence homology and identify the isolates.

Uniplex PCR for identification of *V. cholerae*

This assay used to identify *V. cholerae* by using *OmpW* specific primer. It was performed according to [19]. Primer sequences for amplification of *OmpW* gene were shown in table (3), while PCR program for amplifying *OmpW* gene was shown in table (4).

Table (3) Primers sequences for amplification of *OmpW* gene

N.of Primers	Primer Sequences (5′→3′)	Size of product
F	CACCAAGAAGGTGACTTTATTGTG	304bp
R	GGTTTGTCGAATTAGCTTCACC	

Table (4) Polymerase chain reaction program for amplifying *OmpW* gene

Stages	Temperature	Time	Cycle	Size of product
Initial denaturation	94 °C	10 min	1	304bp

Denaturation	94 °C	1 min	30	
Annealing	59 °C	1min.		
Extension	72 °C	2 min.		
Final extension	72 °C	10 min	1	
Cooling	4°C			

Antibiotic susceptibility test

Using of Kirby Bauer disk diffusion method [20]. All *V. cholerae* isolates in this study were examined for antibiotic resistance and the isolates were classified as sensitive, intermediate, or resistant based on the standards that the Clinical and Laboratory Standards Institute (CLSI) has issued [21] were shown in table (5).

Table (5) Antibiotics discs used in this study

NO.	Antibiotics discs	Content	Inhibition zone\diameter (Mm)			Company\ Origin
			S(≥)	I	R (≤)	
1.	Chloramphenicol (C)	30 µg	18	13-17	12	Liofilchem / Italy
2.	Trimethoprim-sulfamethoxazole (SXT)	25 µg	16	11-15	10	
3.	Cefepime (FEP)	30 µg	18	15-17	14	
4.	Ciprofloxacin (CIP)	5 µg	21	16-20	15	
5.	Gentamicin (GN)	10 µg	15	13-14	12	
6.	Nalidixic acid (NA)	30 µg	19	16-18	19	
7.	Erythromycin (E)	15 µg	22	15-21	14	
8.	Tetracycline (TE)	30 µg	19	15-18	14	
9.	Ampicillin (AMP)	10µg	17	14-16	13	

10.	Piperacillin-Tazobactam (PTZ)	36 μg	22	18-21	17	

Result

Isolation and characterization of *Vibrio cholerae*

Various methods were used in this study to diagnose *Vibrio cholerae,* in biochemical test, out of total 400 stool samples, only 45 isolates were identified as *Vibrio cholerae* and API 20E tests showed 41 isolates as *Vibrio* while molecular method showed 38 isolates as *Vibrio cholerae*. While 50 water samples examined in current study, revealed 3 isolates by routine biochemical test, only one isolate by API 20 E test, while molecular method did not reveal the appearance of *Vibrio cholerae*, table (6).

Table (6) Comparison of diagnostic methods to *Vibrio cholerae* in stool and water samples

Types of methods	Number of water examined	Percentage % of isolates	Number of stool examined	Percentage % of isolates
Biochemical test	50	3(6%)	400	45(11.25%)
API 20 E test	50	1(2%)	400	41(10.25%)
Molecular method	1	0(0%)	41	38(92.68 %)

Serological test

All isolates in the current study were detected as serogroup O1 (*V. cholerae*), serotype Ogawa .

Molecular detection of *V. cholerae*

The findings of isolation process from 41 isolates diagnosed by API 20 E test showed that 38 isolates with 92,68 % were confirmed by *16S rDNA* as *V. cholerae* were shown in figure (1).

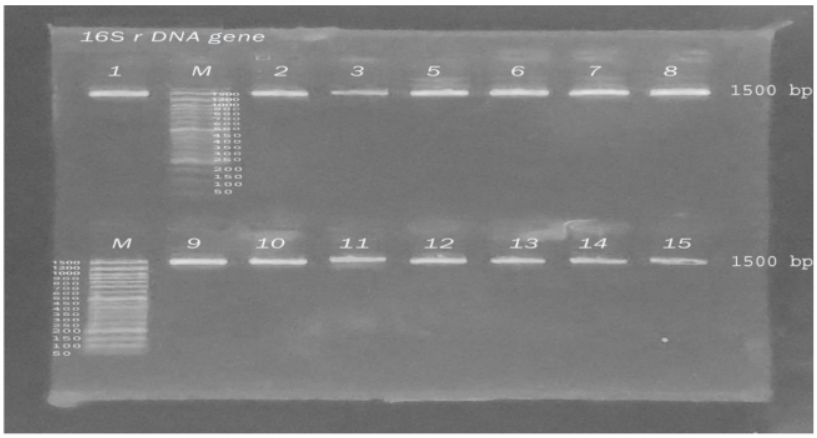

Figure (1) Agarose gel electrophoresis for PCR products of *16S rDNA*(1500bp) from genomic DNA of *Vibrio cholerae*

Recording of new bacterial strains

In the present study, the isolates were identified with 100% similarity, except two isolates were with <100% similarity including LC793854 and LC793858 were recorded as new isolates, were shown in (table 7).

Table (7) Isolates of *V. cholerae* with accession numbers submitted at Gen Bank (NCBI)

Samples No.	Accession Number (Sequence size)	Percentage Identification %	organisms	References copies (NCBI)
1	LC793823 (1348 bp)	100%	*Vibrio cholerae*	CP053810.1
2	LC793824 (1348 bp)	100%	*Vibrio cholerae*	CP053810.1
3	LC793825 (1348 bp)	100%	*Vibrio cholerae*	CP053810.1
5	LC793826 (1348 bp)	100%	*Vibrio cholerae*	CP053810.1
6	LC793827 (1348 bp)	100%	*Vibrio cholerae*	CP053808.1
7	LC793828 (1348 bp)	100%	*Vibrio cholerae*	CP053808.1
8	LC793829 (1348 bp)	100%	*Vibrio cholerae*	CP053810.1
9	LC793830 (1348 bp)	100%	*Vibrio cholerae*	CP053810.1
10	LC793831 (1348 bp)	100%	*Vibrio cholerae*	CP053810.1
11	LC793832 (1348 bp)	100%	*Vibrio cholerae*	CP053810.1

12	LC793833 (1348 bp)	100%	*Vibrio cholerae*	CP053810.1
13	LC793834 (1348 bp)	100%	*Vibrio cholerae*	CP053810.1
14	LC793835 (1348 bp)	100%	*Vibrio cholerae*	CP053810.1
15	LC793836 (1348 bp)	100%	*Vibrio cholerae*	CP053810.1
16	LC793837 (1348 bp)	100%	*Vibrio cholerae*	CP053810.1
17	LC793842 (1348 bp)	100%	*Vibrio cholerae*	CP053810.1
18	LC793843 (1348 bp)	100%	*Vibrio cholerae*	CP047059.1
19	LC793844 (1348 bp)	100%	*Vibrio cholerae*	CP053810.1
20	LC793845 (1348 bp)	100%	*Vibrio cholerae*	CP053808.1
21	LC793846 (1348 bp)	100%	*Vibrio cholerae*	CP053808.1
22	LC793847 (1348 bp)	100%	*Vibrio cholerae*	CP053810.1
23	LC793848 (1348 bp)	100%	*Vibrio cholerae*	CP053810.1
24	LC793849 (1348 bp)	100%	*Vibrio cholerae*	CP053808.1
25	LC793850 (1348 bp)	100%	*Vibrio cholerae*	CP053810.1
26	LC793851 (1348 bp)	100%	*Vibrio cholerae*	CP053794.1
27	LC793852 (1348 bp)	100%	*Vibrio cholerae*	CP053808.1
28	LC793853 (1348 bp)	100%	*Vibrio cholerae*	CP053810.1
29	LC793854 (1348 bp)	99.93 %	*Vibrio cholerae*	CP053794.1
30	LC793855 (1348 bp)	100%	*Vibrio cholerae*	CP053810.1
31	LC793856 (1348 bp)	100%	*Vibrio cholerae*	CP053808.1
32	LC793857 (1348 bp)	100%	*Vibrio cholerae*	CP053808.1
34	LC793858 (1348 bp)	90.70 %	*Vibrio cholerae*	CP053796.1
35	LC793859 (1348 bp)	100%	*Vibrio cholerae*	CP053810.1
38	LC793860 (1348 bp)	100%	*Vibrio cholerae*	CP047295.1
40	LC793838 (1348 bp)	100%	*Vibrio cholerae*	CP053808.1
41	LC793839	100%	*Vibrio cholerae*	CP053808.1

	(1348 bp)			
42	LC793840 (1348 bp)	100%	*Vibrio cholerae*	CP053808.1
43	LC793841 (1348 bp)	100%	*Vibrio cholerae*	CP028827.1

Molecular detection of specific gene in *V. cholerae*

The particular gene (*OmpW*), which is typically present in 38 isolates of *V. cholerae* that were diagnosed in present study (figure 2 and table 8).

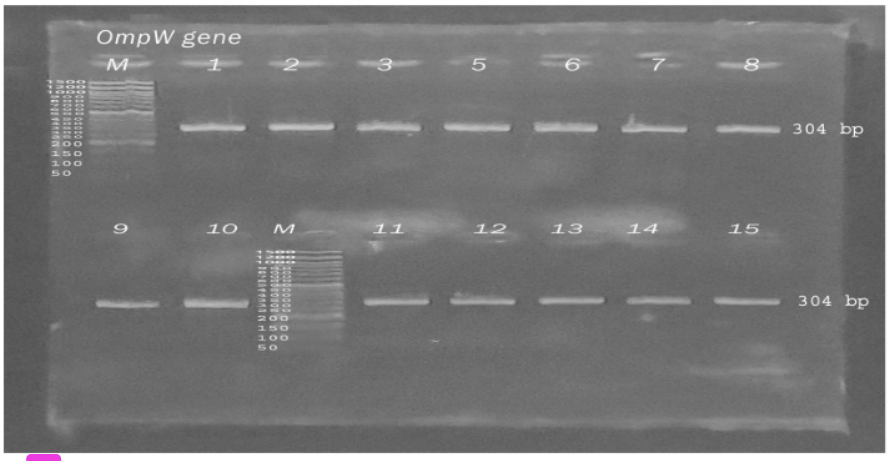

Figure (2) Agarose gel electrophoresis for PCR products (304 bp) of *OmpW* gene of *V. cholerae*

Table (8) Percentage of genes presence in isolates that were diagnosed as *Vibrio cholerae*

Type of genes	Examined isolates	Diagnosed isolates	Percentage % of genes
16SrDNA	41	38	92.68 %
OmpW	41	38	92.68 %

- Whereas 41 isolates were identified as *Vibrio cholerae* by API 20 E

Antibiotic sensitivity test

Vibrio cholerae were tested for antibiotic susceptibility, in current study, it was used different antibiotics and results revealed highest percentage rate of resistance was (100%) to trimethoprim-sulfamethoxazole while lowest percentage rate of resistance (5.26%) to piperacillin-tazobactam were shown in table (9) and figure (3), (4).

Table (9) Percentage of *V. cholerae* susceptibility to antibiotics

Antibiotics	**R** **No (%)**	**I** **No (%)**	**S** **No (%)**
Trimethoprim-sulfamethoxazole (SXT)	38(100%)	0(0%)	0(0%)
Chloramphenicol (C)	34(89.47)	2(5.26%)	2(5.26%)
Nalidixic acid (NA)	34(89.47%)	4(10.52%)	0(0%)
Ampicillin (AMP)	34(89.47%)	2(5.26 %)	2(5.26 %)
Gentamicin (GN)	24(63.16%)	10(26.31%)	4(10.52%)
Erythromycin (E)	24(63.16%)	14(36.84%)	0(0%)
Tetracycline (TE)	17(44.74%)	17(44.74%)	4(10.52%)
Ciprofloxacin (CIP)	4(10.52%)	30(78.95%)	4(10.52%)
Cefepime (FEP)	4(10.52%)	10(26.31%)	24(63.16%)
Piperacillin-Tazobactam (PTZ)	2(5.26 %)	5(13.16 %)	31(81.58 %)
Chi-square =315.359	Df=18	P. value <0.01	

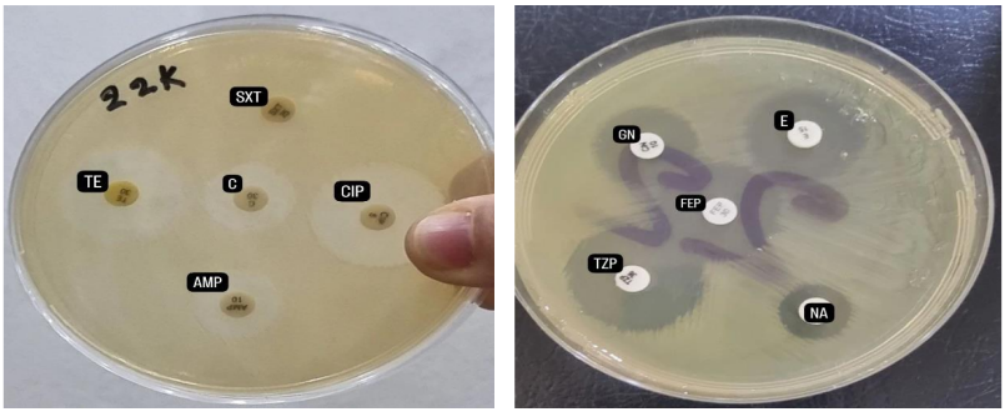

Figure (3) Sensitivity test of *Vibrio cholerae* to antibiotics.

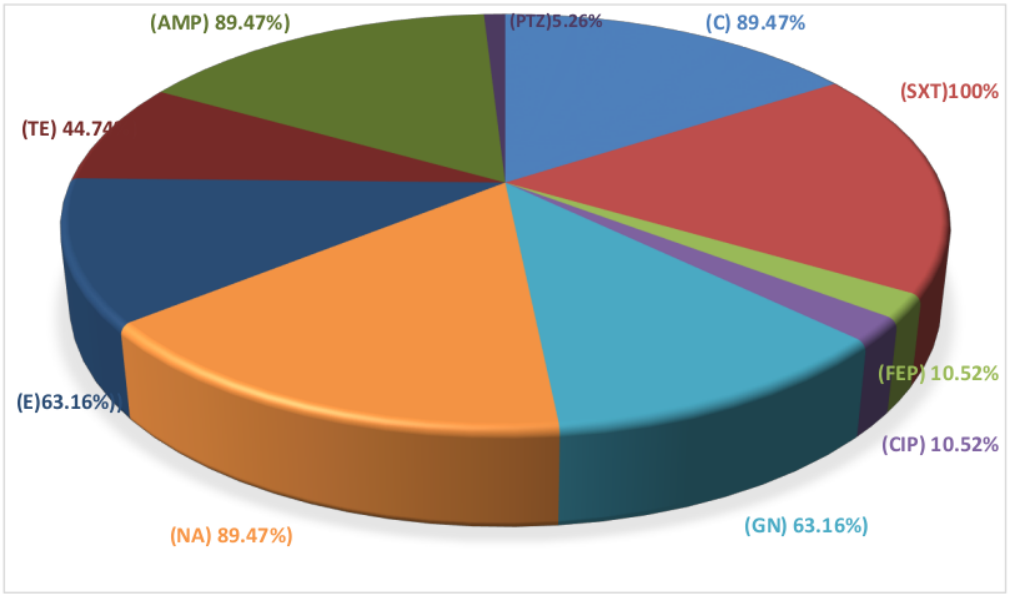

Figure (4) Resistance of *Vibrio cholerae* to antibiotics that included in this study

Discussion

One of the main causes of fatal diarrhea is *Vibrio cholerae,* the microbe is consumed through contaminated food and water, resulting in the illness [22].

The present study revealed 38 isolates out of 41 isolates diagnosed by *16S rDNA* which was in agreement with [27]. Certain *Vibrio* species can be challenging due to differences in their biochemical features, hence all biochemical identifications should

be verified using molecular techniques [28]. Moreover bacteria, has stable regions that enable bacterial identification and other variable regions that enable the differentiation of various bacterial species and mutations in this gene do not affect its basic role of diagnosis. For these reasons, *16S rDNA* gene is the gold standard for diagnosing bacteria[10]. The similarity in the results indicates polymerase chain reaction is a highly sensitive molecular technique [29]. The current study revealed no *Vibrio cholerae* appearance in water samples, even though *V. cholerae* and other vibrios are inhabitants of water bodies, their numbers remain low for testing purpose which was agreement with (Daboul *et al*., 2020) did not reveal of *V.cholerae* O1 strains are known to be difficult to isolate.

All isolates that obtained in the present study were isolates from stool samples identified as serotype Ogawa *Vibrio cholerae* O1 serogroup, which was in agreement with [23] who reported that Ogawa as the predominant serotype of *V. cholerae* O1, the etiological agent of the cholera epidemic in the Sulaymaniyah province in 2022 and agree with[24] in Lebanon who found all isolates were *V. cholerae* O1, serotype Ogawa.

The prevalence of serotype Ogawa due to movement from the northern regions of Iraq as a result of tourism activity in the summer [25], or may due to the extended impending Al-Arbaeen religious ceremonies and other religious festivals, which bring millions of people from all over the world together in a single small city, the cholera outbreaks, especially in Iraq, may have an international impact. People exchange food and slaughter animals on certain days as part of the sacred Al-Arbaeen ceremony [26]. That there is may be an association between cholera occurrence and large family size compatible with the family structure of Thi-Qar province, where the large of the households are made up of many families sharing a single home and toilet.

The appearance of *OmpW* specific gene in all *Vibrio choleare* isolates. It should be mentioned that under the used experimental circumstances, no amplified product was produced by any of the bacterial strains other than *V. cholerae* which was match with[30] and [19]. Who found gene of *OmpW* in all O1 isolates of *V. cholerae*. *Vibrio cholerae* is resistant to environmental shocks like as pH change, osmotic stress, and detergents because of the extramembrane protein *OmpW*. Furthermore, this protein makes it possible for *V. cholerae* to invade the gut and cause illness [31].

Results of present study indicate all 38 isolates were resistance to chloramphenicol, gentamycin, ampicillin, nalidixic acid, erythromycin and trimethoprim-sulfamethoxazole which was disagreement with [32] who found that all isolates were

sensitive to gentamycin, chloramphenicol and agree with [33] who found all isolates resistant to ampicillin, nalidixic acid, erythromycin and trimethoprim-sulfamethoxazole. One of the most important problems for world health is antibiotic resistance, the primary cause of the growing resistance is the easy access to antibiotics and their extensive use in chemoprophylaxis, which leads to resistance [34].

The current study showed all isolates were sensitive to beta-lactams piperacillin-tazobactam which was in agreement with [35]. The effectiveness of this drug may be due to its not being widely used against *Vibrio cholerae*.

Conclusion

Ogawa was the predominant serotype of *V. cholerae* O1, which was the etiological agent of the cholera epidemic in Thi-Qar province in 2023. The gene *OmpW* has high accuracy to diagnose *Vibrio cholerae,* no less important than *16S rDNA*. This study demonstrates the increasing multidrug resistance of *V. cholerae* isolates to routinely used medications, particularly to trimethoprim-sulfamethoxazole 100%. All isolates were sensitive to beta-lactams piperacillin-tazobactam and it is recommended to add it to the Antimicrobial Susceptibility Testing Guidelines.

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