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A Molecular and Epidemiological Study of Cholera Outbreak in Sulaymaniyah Province, Iraq, in 2022

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Abstract

Cholera is a disease caused by a Gram-negative bacterium *Vibrio cholerae* and is among the significant threats to global public health. The disease is mainly spread in the hot months of the year; low sanitation and lack of clean water are the major causes of the disease. In this study, we conducted a molecular and epidemiological study of the recent outbreak in the city of Sulaymaniyah in Iraq. Based on the bacteriological, serological, and molecular identification of the bacterium, it was shown that *V. cholerae* O1 serotype Ogawa caused the disease. Additionally, the number of positive cholera cases were higher in June compared to July (391 positive cases in June and 23 in July). Moreover, the majority (>60%) of the cholera cases were recorded among 20–44-year-old people in both months; however, there was no significant difference in the patient genders diagnosed every month. Overall, this is the first report on the recent cholera outbreak in the city of Sulaimaniyah in Iraq.

K e y w o r d s: *Vibrio cholerae*, outbreak, bacterial infection, molecular identification, Sulaymaniyah

Introduction

Cholera is an enteric bacterial infectious disease caused by a Gram-negative, motile, flagellated bacterium *Vibrio cholerae* (Harris et al. 2012). The infection occurs from consuming vegetables and/or drinking water contaminated with the pathogen (Harris et al. 2012). The disease is particularly a problem in developing countries where poor sanitation, and lack of awareness and/or access to clean drinking water exist (Ali et al. 2015; Ramazanzadeh et al. 2015).

More than 200 serogroups of the bacteria have been identified so far, and cholera outbreaks mainly occur during the hot months of summer elicited by the O1 or O139 biotype (Ali et al. 2015; Ramazanzadeh et al. 2015). Severe watery diarrhea, known as "rice-watery" stool, odorless or of a mild fishy smell, with or without vomiting, are the major symptoms of the disease (Harris et al. 2012; Endris et al. 2022). Among different age groups, children below five years old are the most vulnerable to cholera compared to adults or the elderly (Deen et al. 2008). Additionally, people suffering from other diseases such as gastric disease and/or those individuals with type-O blood groups are at higher risk of getting cholera than other blood group types (Braunwald et al. 2001).

Cholera is considered a global health issue, and it has affected most parts of the world since the early of the nineteenth century (Harris et al. 2012). A majority (~99%) of cholera cases have been recorded in Africa and Southern Asia (Deen et al. 2008; Endris et al. 2022). In 2006, more than two million cholera cases with 6,311 deaths were recorded in 52 countries (WHO 2007a). Since 2020, 323,320 cholera cases have been reported in 27 countries, with 857 deaths (WHO 2021). In Iraq, the first recorded cholera epidemic dates back to 1820 in Basra, which resulted in many deaths (Al-Abbassi et al. 2005). Since 1966, cholera has been

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considered an endemic disease in different parts of Iraq (Hussain and Lafta 2019). In 2007, a study was conducted among 6,399 children suspected of cholera in the city of Kirkuk-Iraq and showed that 326 (5.1%) of them were infected with *V. cholerae*, of which five had died from the infection (Noaman et al. 2011). In another study, 4,667 cholera cases were reported in Iraq in 2007; 136 (2.9%) of them were recorded in the capital city of Baghdad, and about 33% of the confirmed cases were individuals between the age of (0–4) and (15–45) years old (Khwaif et al. 2010).

In 2022, a new cholera outbreak hit different cities of Iraq. According to the official data of the World Health Organization (WHO), 449 confirmed cholera cases were recorded in Iraq (WHO 2022). Here, we report the cholera outbreak data analysis and molecular identification of the pathogen in Sulaymaniyah from the beginning of June to the end of July 2022.

Experimental

Materials and Methods

The current molecular and epidemiological study was carried out on cholera cases that spread in Sulaymaniyah city, Iraq from the 1st of June 2022 to the end of July 2022. Samples and data were collected in the Shar Hospital.

Sample collections. Stool samples were collected according to the Iraqi Standard Operating Procedures for Laboratory Identification of *Vibrio cholerae* (USAID, SOP: NCL – BE 00110) (SOP 2014). In brief, stool samples were collected from suspected patients in the Cary-Blair transport medium (Sun et al. 2020). Samples were transferred to alkaline peptone water (APW, pH = 8.6) and kept for 4 to 6 hours at 37°C to identify the pathogen using bacteriological, biochemical, serological, and molecular biology methods.

Bacterial identification and biochemical tests. Colony characterization of the bacterium was carried out by sub-culturing a single colony from APW onto a selective media of thiosulfate-citrate-bile salts-sucrose (TCBS) agar and incubated at 37°C for 18–24 hours. The colonies were isolated and subjected to the following biochemical tests: oxidase test (Shields and Cathcart 2010) and Kligler's Iron Agar (KIA; Thermofisher, UK) test.

Serologic identification of *V. cholerae*. Slide agglutination test with monovalent O1 or O139 antisera (Mast group, UK) was used for serological identification of *V. cholerae* following the manufacturer's instructions. In brief, freshly grown colonies were tested against *V. cholerae* O1 and O139 serogroups monovalent antiserum. Isolates agglutinating in monovalent antiserum to the O1 serogroup were identified as *V. cholerae* O1. To identify the serotype of the serogroup, *V. cholerae* O1 was further tested for agglutination in the monovalent Ogawa and Inaba antisera.

Molecular identification of *V. cholerae*. Molecular identification of the isolates was carried out by amplifying the 16S rRNA gene following previously described protocol (Mohamedsalih et al. 2020). In brief, the bacterial DNA of three confirmed isolates randomly selected were extracted using Prlesto™ Mini gDNA Bacteria Kit (Geneaid, Taiwan). PCR reaction mix (50 µl) contained 0.5 µM of universal 16S rRNA primers (F: 5'-AGAGTTTGATYMTGGCTCAG-3', R: 5'-ACGGYTACCTTGTTACGACTT-3') (Satokari et al. 2001), and 2 × Master mix Prime Taq Premix (GeNet Bio, South Korea). The PCR condition was as follows: initial denaturation (95°C for 2 minutes), followed by 35 cycles of denaturation (95°C for 30 seconds), annealing (58°C for 30 seconds), and extension (72°C, 30 seconds). The final extension period was for 10 min at 72°C before cooling and keeping the sample at 4°C. Aliquots (10 µl) of the amplicons were analyzed by agarose gel electrophoresis and visualized under UV light. The remaining 40 µl of PCR products were sequenced using the Sanger DNA sequencing method (Macrogen, South Korea). Analyzing and assembling the sequences were performed using Chromas software v1.0 (Technelysium software). The assembled DNA sequences were submitted to GenBank, and the accession number for each of the sequences was received using BankIt webpage (https://www.ncbi.nlm.nih.gov/WebSub).

Phylogenetic analysis. The phylogenetic tree was constructed following the method described previously by Chong et al. (2014). Assembled PCR sequences of each of the isolates were compared with closely related 16S rRNA sequences in the NCBI databank using the BLAST homology sequence searching. Multiple sequence alignment was built using ClustalW, and the tree was constructed using MEGA 7 software (Kumar et al. 2016), neighbor-joining method, the bootstrap method (1,000 replica), and the Kimura 2-parameter model.

Statistical analyses. Statistical analysis was performed using XLSTAT software (2019.2.2). The Mann-Whitney *U*-test was used to compare June and July data and analyze the differences between males and females admitted to the hospital each month. Differences were considered significant when the p-value was < 0.05.

Results

Isolation and identification of the pathogen using biochemical and serological tests. All the 414 positive cases recorded within two months study were confirmed using bacteriological and serological tests. Initially, isolation and preliminary identification of *V. cholerae* were

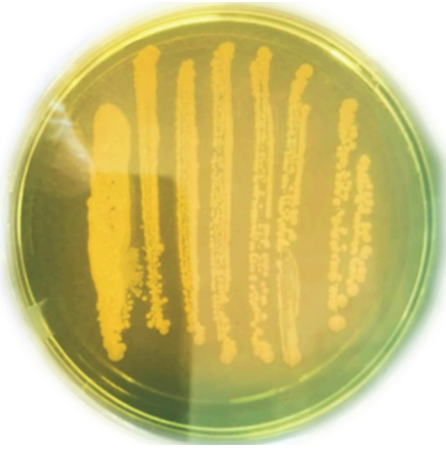


Fig. 1. An example of *Vibrio cholerae* grown on TCBS media.

carried out by culturing aliquot from APW on the TCBS media and checking for the characteristic colonies of *V. cholerae*. The preliminary indications of the *V. cholerae* were yellow, shiny, round, smooth, and slightly flattened colonies on the TCBS media (Fig. 1).

To identify the bacteria the oxidase test, KIA test, and serological tests were used. The *V. cholerae* isolates were oxidase positive (Fig. 2A); acid/alkaline, did not produce gas nor H_2S in the KIA test (Fig. 2B). All the positive tested samples were agglutination positive with monovalent O1 antiserum. The biotype O139 was not detected among the tested samples, and the majority of the samples reacted to monovalent Ogawa antiserum (Fig. 2C).

Identification of *V. cholerae* using molecular and phylogenetic analysis. The partial sequences of the 16S rRNA genes from three random bacterial isolates were used for molecular identification of the etiological agent of the outbreak. DNA sequencing and phylogenetic analysis revealed that the isolates belonged to *V. cholerae* species. The partial sequences of the three isolates were submitted to the GenBank with the following strain names: *V. cholerae* strain Suli1 (Accession number: OP470022), *V. cholerae* strain Suli2 (Accession number: OP470023), and *V. cholerae* strain Suli3 (Accession number: OP470024). Additionally, the phylogenetic tree showed that the 16S rRNA sequences of the isolates formed a clade with other *V. cholerae* strain ATCC® 14035™ (NR 119302), *V. cholerae* strain CECT 514 (NR 044853), and *V. cholerae* strain RC782 (NR 044050) (Fig. 3).

Table I
Percentage distribution of diagnosed cases according to age groups and gender for both June and July 2022.

Age group (years)	Gender	June		July	
		Number of cases	%	Number of cases	%
<1	Male	0	0.00%	0	0.00%
	Female	0	0.00%	0	0.00%
1–4	Male	0	0.00%	0	0.00%
	Female	1	0.00%	0	0.00%
5–9	Male	4	0.10%	0	0.00%
	Female	0	0.00%	0	0.00%
10–14	Male	9	0.30%	3	0.20%
	Female	25	0.70%	15	1.20%
15–19	Male	149	4.30%	66	5.20%
	Female	217	6.20%	95	7.40%
20–44	Male	1,156	33.30%	412	32.20%
	Female	980	28.20%	379	29.60%
45–64	Male	340	9.80%	90	7.00%
	Female	381	11.00%	131	10.20%
>65	Male	97	2.80%	25	2.00%
	Female	115	3.30%	64	5.00%
Total cases		3,474	100%	1,280	100%

Epidemiology of the disease. During two months of the study, 4,754 suspected cases were admitted to the Shar hospital/Sulaymaniyah city, Iraq (3,474 cases in June and 1,280 in July). The percentage of males to females in June and July were 51%:49% and 47%:53%, respectively. It was noticed that the majority of the patients were 20–44 years old (61.5% in June and 61.8% in July) (Table I). The number and the percentage of

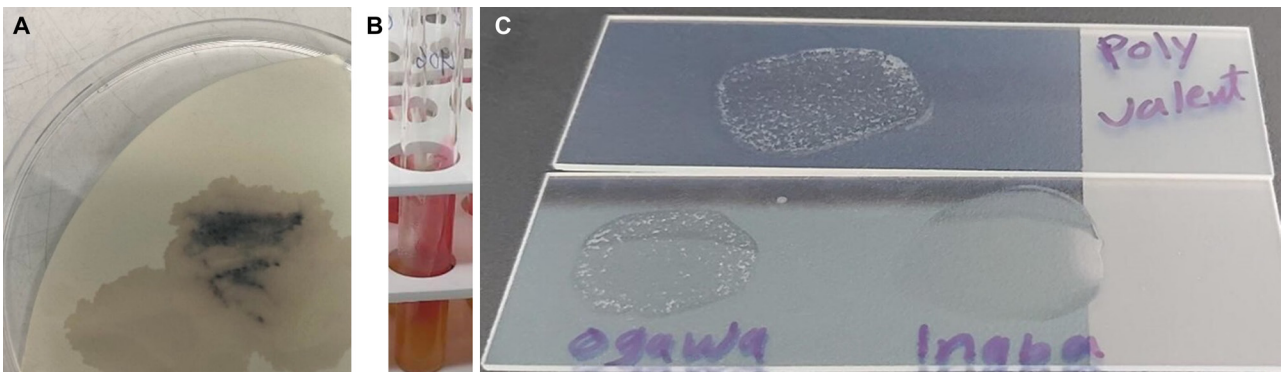


Fig. 2. The result of the bacteriological identification of *Vibrio cholerae*.
A) In the oxidase test, the purple color indicates a positive result; B) KIA test, acid/alkaline, no gas, no H_2S; C) serologic identification of *V. cholerae*, showing agglutination for polyvalent O1 antiserum and monovalent Ogawa.

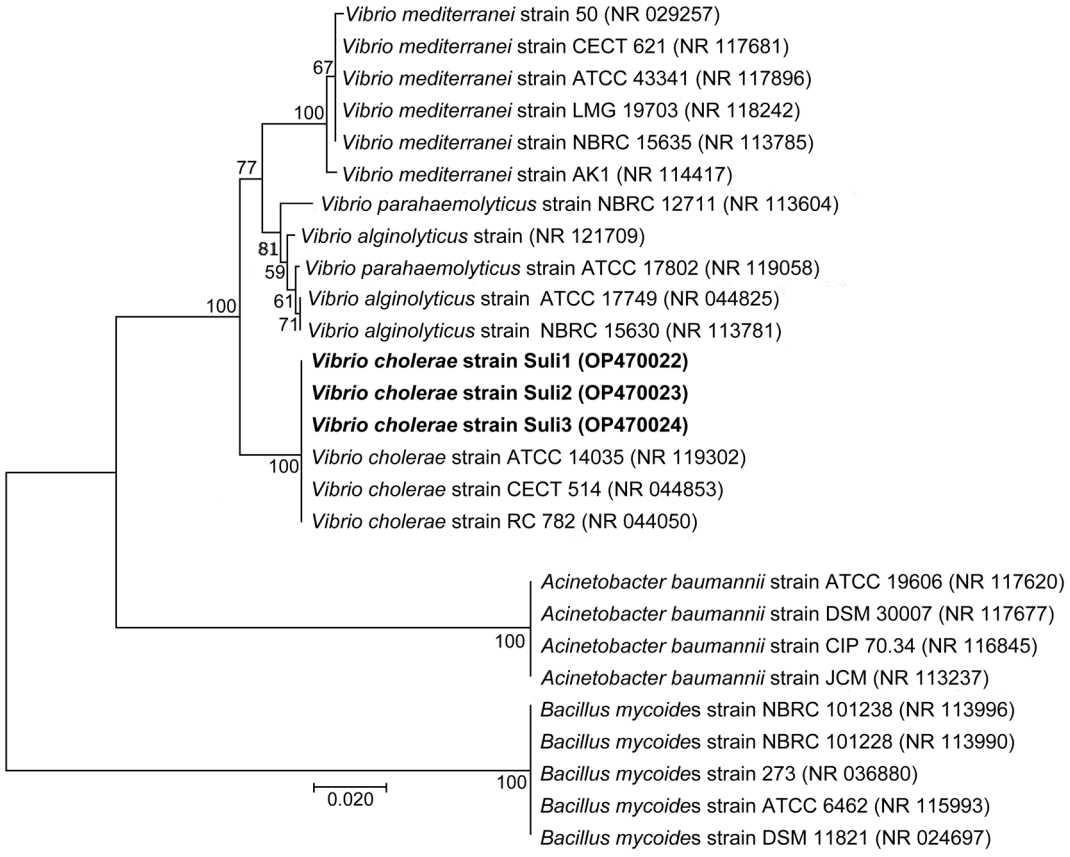


Fig. 3. The phylogenetic tree analysis is based on the similarity of the 16S rRNA sequence of the newly isolated *Vibrio cholerae* to closely related species. The tree was built using MEGA7.0 software, the neighbor-joining method, and bootstrapped 1,000 replicate runs. The newly isolated are shown in bold, and the accession numbers are shown in parentheses after the strain names. The bar indicates substitutions per nucleotide.

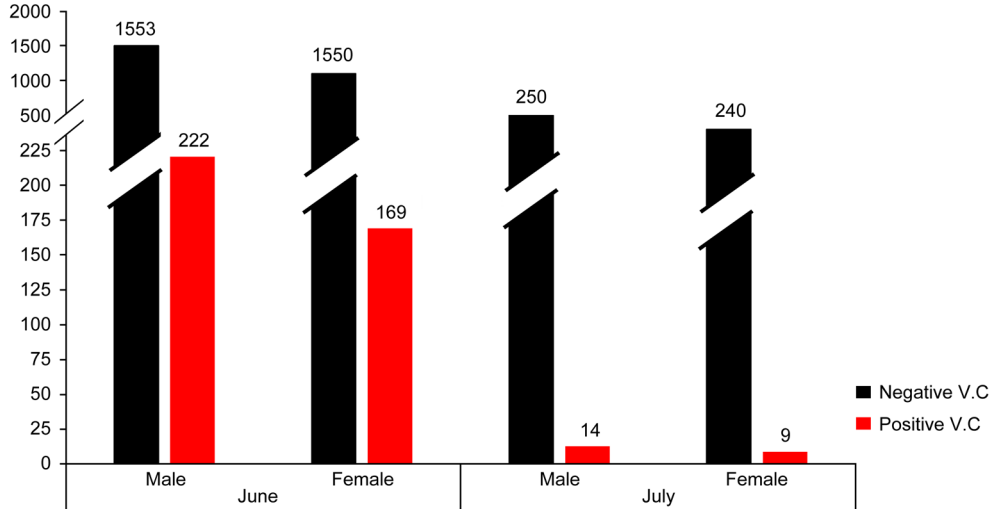


Fig. 4. The number of suspected and positive cholera cases in the Shar hospital for both genders in June and July of 2022.

the suspected age groups are shown in Table I. Out of 3,474 suspected cholera cases in June, 391 (11.3%) were diagnosed as cholera-positive, of which 222 were males, and 169 were females. Whereas the number of positive cases decreased to only 23 (1.8%) cases out of 1,280 suspected cholera cases in July of which 14 of them were male, and nine were female (Fig. 4).

The mean and the standard deviation of the male and female cholera cases were 56.61 ± 52.8 and 55.4 ± 46.37 in June, respectively. In July, the mean and the standard deviation data were 19.22 ± 7.54 and 22.06 ± 10.3 for males and females, respectively.

Statistically, the result (Mann-Whiney *U*-test) of the comparison between both genders for each

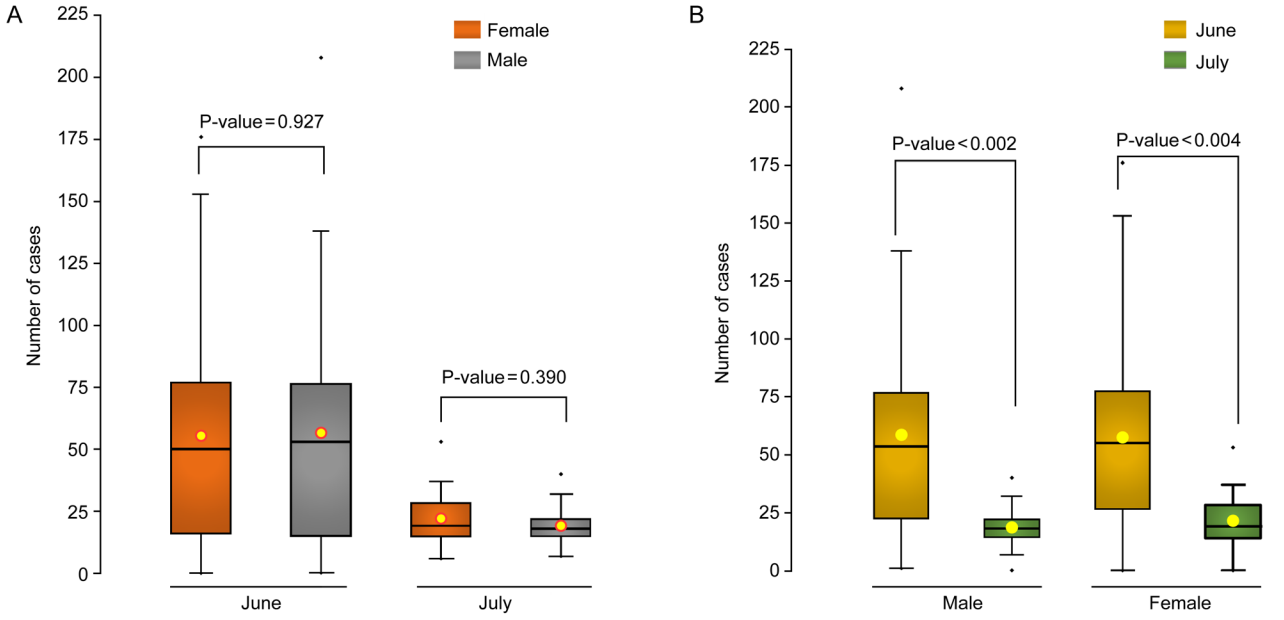


Fig. 5. Box plot showing the number of diagnosed patients admitted to the hospital based on gender; A) comparison between both genders for each month; B) differences between the number of male cases in June and July as well as female cases in June and July. The values' statistical significance (p-value) is shown above each box. The yellow dot in each box plot represents the mean value, and the horizontal line in each box represents the median value. A p-value < 0.05 is considered significant.

month showed that there is no significant difference (p-value > 0.05) among the genders (Fig. 5A).

The data of male and female genders that were admitted into the hospital in both months were further analyzed for both June and July. The results have demonstrated that there was a significant difference (p-value < 0.05) in the number of males in June and July as well as the number of females in June and July that were admitted to the hospital (Fig. 5B). Furthermore, the mean and standard deviation for the number of males in June and July were 58.50 ± 52.64 and 18.62 ± 8.16 respectively; and for females in June and July, were 57.30 ± 45.99 and 21.37 ± 10.86, respectively.

Discussion

Cholera is among the serious global health issues, particularly in Africa and southern Asia; according to WHO, it infects around 1.3 to 4 million people and causes between 21,000 to 143,000 deaths worldwide each year (Chowdhury et al. 2022). Several cholera outbreaks have been recorded in Iraq; the latest one was started at the end of May 2022, and according to a WHO report, 449 confirmed cases in Iraq by 24 July 2022 (WHO 2022). Here, we carried out a molecular and epidemiological study of the latest cholera outbreak in the Sulaymaniyah province of Iraq for the first time. The data presented here were from the public Shar hospital during the outbreak peak from the beginning of June to the end of July 2022.

Bacteriological, biochemical, and molecular identification of the isolates confirmed that all the isolates from this study belonged to the same biotype. Therefore, the etiological agent of the cholera outbreak in the Sulaymaniyah province in 2022 was *V. cholerae* biotype O1, and Ogawa was a dominant serotype. Preliminary identification showed that these bacteria were oxidase positive and changed the TCBS media's color from green to yellow; they produced acid/base, but did not produce gas nor H_2S in the KIA test (Swanson and Gillmore 1964; Lányi 1988). Moreover, serological tests revealed that isolates belonged to biotype O1, and Ogawa was a dominant serotype. Previously, it has been reported that the cholera outbreak of 2015 in Baghdad was caused by *V. cholerae* O1, biotype El Tor, and serotype Inaba (Jameel et al. 2016). Another study of 80 clinical and five environmental *V. cholerae* isolates from the outbreak in Iraq in 2007–2009 revealed that 55% of clinical isolates belonged to the Inaba serotype, 32.5% to the Ogawa serotype, and 12.5% to the Non-O1 serotype, while all the environmental isolates belonged to the Non-O1 serotype (Saleh et al. 2011). In 1995, WHO reported a cholera outbreak in Sulaymaniyah governorate that resulted in 264 confirmed cases and three deaths. The etiological agent was identified as *V. cholerae* O1 serotype Ogawa (WHO 1995). A comprehensive review of the cholera outbreaks in the Kurdistan region covering seven cholera outbreaks between 1995 and 2022 indicated that both Inaba and Ogawa serotypes of *V. cholerae*, O1, El Tor individually caused these outbreaks (Sidiq 2022). In the outbreaks in 1995, 1998, 1999, and 2012 in Iraq and the

Kurdistan Region the serotype Ogawa was dominant, while only the outbreaks of 2007 and 2015 were caused by the Inaba serotype (WHO 1995; Yassin 2002; Al-Abbassi et al. 2005; Kami 2007; WHO 2007b; Agha et al. 2008; Health Cluster 2015; Zgheir et al. 2019).

Molecular identification of the three randomly selected isolates revealed that these isolates were *V. cholerae*, and all three isolates were closely related to each other and also to *V. cholerae* strains ATCC® 14035™ (NR_119302), CECT 514 (NR_044853), and RC782 (NR_044050). The high sequence identity (> 99.9%) of the randomly selected *V. cholerae* isolates could suggest a common source origin of the pathogen before spreading in the city. Since no environmental sampling was tested in this study, the exact source of the infections remains to be determined. However, based on experimental data reported by WHO on 24 July, 11% of the water samples collected from different sources in Iraq were contaminated with *V. cholerae* (WHO 2022).

Additionally, the polluted water of the Tanjero River, which has been used to irrigate leafy vegetables in the region, can be a possible source of infection. Various waste materials and pollutants are constantly dumped into the Tanjero River in Sulaymaniyah, including sewage, industrial, and agricultural wastes (Aziz et al. 2012; Rahman et al. 2021). Previous studies have demonstrated that polluted lakes, rivers, and food were sources of cholera outbreaks in different regions of the world (Albert et al. 1997; Griffith et al. 2006; Bompangue et al. 2008; Dinede et al. 2020). It supports the hypothesis further that using polluted water of the Tanjero River for irrigation could have been the cause of the cholera outbreak in Sulaymaniyah.

The epidemiological data demonstrated that the number of people admitted to the hospital with cholera symptoms was significantly higher in June than in July: 3,474 and 1,280, respectively. The number of confirmed cholera-positive cases was also higher in June than in July: 391 and 23, respectively. Generally, acute diarrhea and vomiting conditions among people are more prevalent during summer due to contaminated food and drinking water usage. People drink more water, possibly contaminated, during summer to replace lost fluid because of the hot weather, which can also favor the growth of microorganisms on foods and cause food poisoning (Ramos-Alvarez and Sabin 1958; Anderson et al. 2020). Additionally, cholera outbreaks in summer could be due to the hot atmospheric air and low water level, which provide perfect conditions for bacterial growth (Asadgol et al. 2019). However, decreasing the number of cholera case in July could be due to the governmental action plan against the outbreak, such as prohibiting using contaminated products, treating contaminated water sources, and raising public awareness (Jensen et al. 2006; Panja et al. 2016).

Moreover, lytic bacteriophages can destroy the bacteria soon after being discharged into the water following their spread (Jensen et al. 2006; Panja et al. 2016). Some studies have found that vibrio phage can limit bacterial density and growth, and the severity of outbreaks can largely depend on the density of the phage (Jensen et al. 2006; Ujah et al. 2015; Panja et al. 2016; Asadgol et al. 2019). The transmission of cholera from person to person is more severe than those acquired from the environment (Harris et al. 2012).

In our study, only a small portion 391 (11.3%), and 23 (1.8%)) of the suspected individuals were confirmed as cholera cases in both June and July, respectively. No death was reported in the 2022 cholera outbreak in the Sulaymaniyah province; however, three deaths were recorded in the whole of Iraq; two of them were in the city of Kirkuk and one in Baghdad (WHO 2022). Besides *V. cholerae,* other pathogens such as *Entoameba histolytica* can be another causative agent of acute diarrhea and amebiasis is an important public health problem worldwide (Hameed et al. 2021). Previously, *E. histolytica* was identified among children suffering from diarrhea in the Kirkuk and Duhok provinces of Iraq (Hussein and Meerkhan 2019; Hameed et al. 2021). In addition to *E. histolytica*, *Giardia lamblia* could be a reason for acute diarrhea, and the parasite has been previously isolated from the Euphrates River in AL-Nasiriyah City in Southern Iraq (Al Jassas et al. 2022). The enteric parasites heavily contaminate the leafy vegetables in Baghdad city (Khalil 2019). Significant reasons for acute diarrhea and cholera outbreak are contaminated water sources, especially from artesian wells, house tanks, shallow wells in mosques, or water from tankers sold to people (WHO 2022). Several factors are involved in cholera's prevalence, including poor sanitation and hygiene, lack of qualified healthcare, poor infrastructure, and contamination of drinking water sources with sewage system (Ujah et al. 2015; Oguttu et al. 2017).

Moreover, our results showed that the numbers of cholera cases were slightly, but not significantly, higher among males than females; people aged 20–44 were more affected by the disease. It could be because people at this age, especially males, are more frequently eating outside, which might have increased their chances of getting exposed to different pathogens, including *V. cholerae*. A study of a cholera outbreak in Uganda in 2011 indicated that cholera cases were higher in males than females by 1.6 times (Bwire et al. 2017). A prospective study in fishing villages in Uganda between 2011–2015 covering ten cholera outbreaks showed that case-fatality ratio was significantly higher among males than females (Bwire et al. 2017).

The recurrent cholera outbreak in Iraq indicates that efforts are needed to establish safe drinking water,

proper sanitation, and treatment of sewage water, strengthen primary health care, establish disease surveillance mechanism, and improve the nation's awareness and preparedness to detect and respond to cholera outbreaks in the future rapidly.

Conclusions

A cholera outbreak is a bacterial disease caused by the Gram-negative bacterium *V. cholerae* after consuming contaminated food and water. Here for the first time, we reported molecular and epidemiological data on the 2022 cholera outbreak in the Sulaymaniyah province that revealed the incidence rate and identified the strain as *V. cholerae* Ogawa serotype. We found that the number of diagnosed people was higher in June compared to July. Also the majority (>60%) of the cholera cases were recorded among people aged 20–44 years in both months. This study provides an insight into the incidence rate of the cholera outbreak in Sulaymaniyah province during June and July 2022. It reveals the causative bacteria strain than can help authorities develop preventive strategies to stop such outbreaks in the future.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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