ORIGINAL ARTICLE

In silico characteristics for re-emerging possibility of *Vibrio cholerae* genotypes in Iran

M. Hajia[1] **and Amir Sohrabi**[2]

1) *Department of Molecular Biology, Research Center of Health Reference Laboratory, Ministry of Health and Medical Education, Tehran, Iran and* 2) *Department of Medical Epidemiology and Biostatistics, Karolinska Institutet, Stockholm, Sweden*

Abstract

Epidemic cholera has been registered several times within recent years in Iran. The dominant genotype was Ogawa until 2011, but this gradually changed to Inaba. However, in 2015, the re-appearance of a previous Ogawa genotype was detected by the Iranian CDC. This raised worries because no evidence was found for its origin abroad. The aim of the present study was to identify clearly the source of this outbreak. Pulsed field gel electrophoresis (PFGE) was used to compare the recently detected *Vibrio cholerae* strains with those isolated from 2011 to 2015. We selected one strain per PFGE pattern, and compared the distinct patterns. BioNumerics software was applied, which enables interpretation of phenotypic and genotypic differences. In total, we studied 33 *V. cholerae* Ogawa strains. Analysis showed that strains could be discriminated on the basis of annual clusters but with a similarity of more than 80%. The highest homology was observed among those isolated each year from 2011 to 2014. In contrast, strains isolated in 2015 also exhibited close correlation with each other but were located in distinct clusters. The analysis also proved genetic variations among some strains. All 2015 strains showed differences with regard to previous genotypes despite some similarities. The new genotypes were probably imported into Iran from neighbouring countries such as Iraq by travellers or contaminated food sources since 2015. However, more investigations are required to identify the exact source of the 2015 outbreak.
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Corresponding author: M. Hajia, Reference Health Laboratory, #48 ,Keykhosro Shahrokh Alley, Zartoshtian Street, Hafez Avenue, P.O. Box: 11316361111, Tehran, Iran.
E-mail: massoudhajia@yahoo.com

Introduction

Cholera is a treatable infectious disease that has remained an important threat to public health due to poor living conditions and sanitation practices in underdeveloped countries [1–4]. It is very important to find the source of infections and to identify whether its spread is as a re-emerging or newly emerging type. Prompt warning is also necessary to prevent the spread of epidemic disease, as well as detecting sources of the infectious agents [5–7]. Typing methods have a critical role in this and they have been developed to include various approaches such as multiple-locus variable-number tandem repeat analysis (MLVA), multilocus sequence typing and whole genome sequencing. None of these methods can be used in Iran at the national level, nor are they applied in international studies [8–10]. Published studies are limited by these typing procedures, but pulsed field gel electrophoresis (PFGE) has been accepted as the reference standard typing technique [11]. This method has been used for several years in Iran, but the images have been analysed using different software (GelCompar) in all previous and some recent studies until 2011 [12–14]. Use of this software is not accepted by the PulseNet international committee [15–17], so the results must be meaningful and interpretable for each analysed image from all results reported by members.

However, PFGE—an efficient molecular typing method for accurate classification of *Vibrio cholerae* isolated types—has

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been applied in Iran and in other member states of PulseNet International. Introducing new molecular typing methods with the use of BioNumerics software has provided great progress in classification and describing of the phylogenic tree of any targeted organism. This method was validated several years ago and has now been standardized for many enteric pathogens [18,19]. Currently, the introduced PulseNet protocol has been accepted by many countries as a highly discriminating typing technique [20]. It is being applied as a critical tool for the monitoring and control of enteric pathogens worldwide [21]. This procedure is used in the Health Reference Laboratory, especially for cholera patients in Iran [22–25].

The dominant genotype in a nationwide *V. cholerae* epidemic outbreak in 2011 was Ogawa. The dominant genotype gradually changed to the Inaba genotype in the epidemic cholera outbreaks that followed and the spread of Ogawa was limited to some local areas. In 2015 Ogawa type was again detected as the dominant type, a fact that alarmed the Iranian surveillance system.

The aim of this study was to find out whether the source of this outbreak was a new emerging genotype or whether it had re-emerged from the previous cholera outbreaks. *In silico* analysis was performed on all the collected PFGE patterns for investigation of re-emerged genotypes from previous epidemics in Iran.

Materials and methods

Confirmation process of cholera infection

Each suspected case of cholera infection is notified and considered as an alert by the Iranian surveillance programme. When the case is confirmed, an alarm is issued for a local outbreak, if a cluster of three or more cases are detected in a specific area.

The *V. cholerae* isolates were diagnosed from sporadic and epidemic cases in the local laboratories of each region The alerting system was based on the identification of any five new cases in any of the local laboratories during the study period. At the next step, the first five newly detected *V. cholerae* specimens were sent to the reference centre for *V. cholerae* via its local laboratory for final confirmation, as well as for susceptibility testing and genotyping. After confirmation of the cases, the health authority issued the notice to all health services throughout the country [5,25,26].

The specimens were diagnosed using standard biochemical and bacteriological tests and examined for specific serogroups by O1 polyvalent and Ogawa/Inaba mono-specific antisera (BD, Becton Dickinson Co., Frankland Lakes, New Jersey, USA) [27–29].

Study population

All collected Ogawa strains identified since 2015 were entered into the study for assessment of their homology with previously determined types from previous outbreaks. One of each strain previously established as identical with a clear pattern was entered into the study. In total, 33 Ogawa strains were studied, including 10 from the year 2011, 6 from the year 2014 and 16 from the year 2015. Unfortunately, we could not access Ogawa strains for 2012 and 2013, except one.

We could not analyse strains isolated before 2011, nor the strains issued from other studies. Besides, their images have been analysed using other software, such as GelCompar software, which could not be integrated into our database.

PFGE method

Genotyping of isolates was performed by PFGE using PulseNet standard procedure for *V. cholerae* specimens [30]. The whole agarose-embedded genomic DNA from *V. cholerae* was prepared and the previously described procedure [24,26,27,31] was carried out. The fingerprinting patterns in the PFGE gels were re-analysed using the computer software package BioNumerics 6.6 (Applied Maths, Keistraat 120. 9830 Sint-Martens-Latem, Belgium), based on the PulseNet SOP. After background subtraction and gel normalization, the fingerprint patterns were subjected to typing on the basis of banding similarity and dissimilarity using the dice similarity coefficient and clustering based on the unweighted-pair group method using average linkages (known as UPGMA), as recommended by the software manufacturer, and results are graphically represented as a dendrogram [19,26]. Results were analysed and interpreted according to the guidelines proposed by Tenover et al. [24,32,33].

Approving of technical performance

This technique is operated in two main steps. First is the preparation of specimens for the test and running the test with a specific machine (CHEF Mapper XA System, Bio-Rad,

TABLE 1. Frequency of Inaba and Ogawa serotypes during study (Mafi et al., 2016 [5])

	2010	2011	2012	2013	2014	Total
Inaba	0 (0.0%)	13 (1.1%)	20 (37.74%)	254 (98.83%)	5 (55.55%)	292 (19.2%)
Ogawa	15 (100%)	1175 (98.9%)	33 (62.24%)	3 (1.17%)	4 (44.45%)	1229 (80.8%)
Total	15 (100%)	1188 (100%)	53 (100%)	257 (100%)	9 (100%)	1521 (100%)

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Hercules, CA, USA), the second is the computer analysis of specimen patterns.

All technical staff were trained during workshops held by EMRO in Cairo from 2006 to 2009. It was decided to run some research projects using this technique in order to acquire experience [14,20,23,24]. After that, the PFGE technique was validated and standardized for food-borne pathogens isolated in 2010. All experiments were performed under the supervision of EMRO and the regional officer in the Health Reference Laboratory during a couple of workshops [16].

PFGE interpretation criteria

Tenover et al. proposed a guideline for PFGE interpretation [33]. Following that guideline, a banding pattern difference of three fragments could occur as the result of a single genetic event, so these isolates have been classified as highly related, differences of four to six restriction fragments are probably due to two genetic events, and differences of more than seven restriction fragments are the result of three or more genetic events. Isolates that differ by three fragments in PFGE analysis may represent epidemiologically related subtypes of the same strain.

Results

Cholera outbreaks generally started in southeast Iran, close to the border with Pakistan, in late spring. They usually lasted a

FIG. 1. Iranian provinces and cities.

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TABLE 2. Frequency and distribution of outbreaks in 2015

City	No. of isolated strains	Serotype	Date
Ahvaz and its suburbs	45	Ogawa	mid May to mid June
Semnan	1	Ogawa	mid May to mid June
Abadan	1	Inaba	early October
Esfahan	6	Inaba	early to mid November
Gonabad	2	Inaba	mid October
Jiroft	1	Inaba	mid October
Karaj	1	Inaba	late October
Qazvin	2	Inaba	October
Qom	6	Inaba	mid to late October
Rasht	1	Inaba	late October
Tehran	3	Inaba	late September

few weeks, but could last until late summer. All recent outbreaks have been limited to a small part of the country and had the sporadic form except the one that occurred in 2011 in Iran [5]. These *V. cholerae* isolates have been genotyped and it has been noted that dominant strains have been changing gradually to Inaba (Table 1). It was demonstrated that these infections had been transmitted by foreign travellers [22,24].

Two different outbreaks in the last year of the study period occurred at least 3 months apart. In the first outbreak, in late spring, 45 Ogawa specimens were detected. They were detected in Ahvaz and its suburb (southwest Iran), although one case of this type was reported in Semnan, a city in central northern Iran (Fig. 1). The second outbreak was observed in the autumn, in a few cities, and comprised 23 cases. This time, all specimens were identified as Inaba type. These strains have been isolated from the following area: central cities (Arak, Qom, Karaj and Tehran), northern part (Golestan), Mashhad (a tourist town in the northeast), Gonabad (an eastern city near the Afghan border), Bushahr (a port town in the south of Iran) and Ahvaz (a city in southwest Iran) (Table 2 and Fig. 1).

All Ogawa types entered into the study have been analysed. They were verified one by one using BioNumerics software to compare their homology and relatedness by

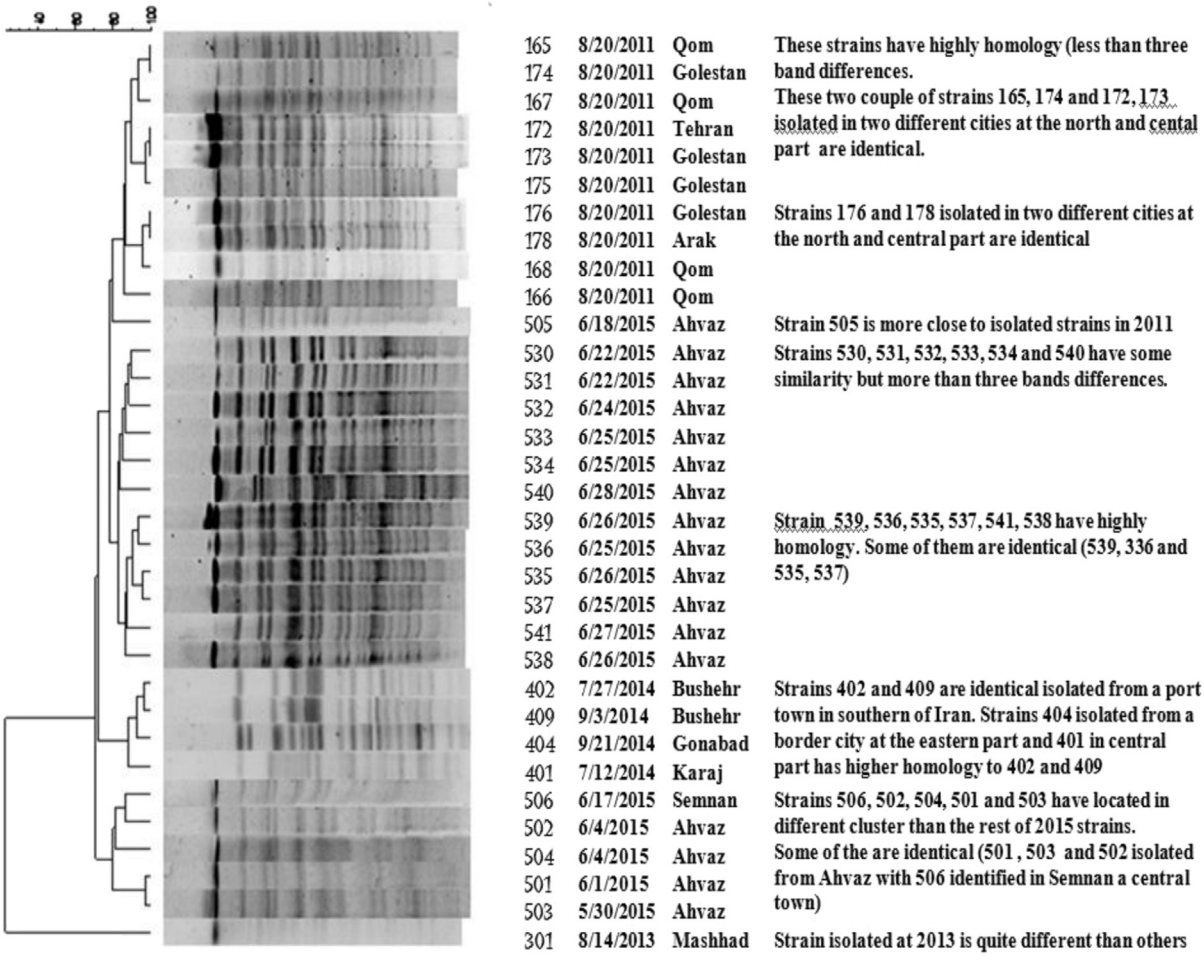

FIG. 2. Dendrogram of analysed Ogawa strains at present study.

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drawing a dendrogram. Repeating this analysis provided similar results.

The studied strains showed high homology, specifically in those isolated each year. The homology of identified strains of the year 2011 was higher than for other years. Strains isolated in 2015 had close correlation with each other but were also located in different clusters (Fig. 2). The analysed images prove genetic variation among some strains (Fig. 2).

Discussion

According to the WHO definition, cholera infection is considered an endemic disease if it has been observed for 3 of the last 5 years [34]. Based on this definition, Iran can be considered as a country where cholera is endemic, at least in some regions. However, in some cases cholera outbreaks have been linked to the cross-border movement of Afghan, Pakistani or other foreign populations. Iranian health authorities have registered outbreaks every year during the study period. Although in previous years, the last case has started near the Iraq border, in Khuzestan Province in southwestern Iran. Following the purpose of this study, which aimed to understand whether the recent outbreak had originated from foreign sources or had come from within the country, we had to compare the homology of recent *V. cholerae* isolates with previously identified accessible strains.

The applied typing technique—PFGE—is able to show how the disease has been spread over the country and whether there is any similarity between different outbreaks. It can also indicate the original source of infection. Analysis of all the collected data showed that each year's outbreak had its own specific pattern with separated clusters. It was also shown that previously identified pulse types were not seen in the next years. The first cholera epidemic that affected the whole country originated from Pakistan [5]. These findings demonstrated that all types analysed in this study were new emergent strains. None of these strains had been registered already. This finding was already proved in previous reports concerning Inaba strains [22].

The dendrogram clearly showed that isolated specimens were all located in different clusters. It was clearly proved that some of the strains analysed in 2015 were identical and located in a highly preserved cluster. The rest of the 2015 types had high similarity but showed some genetic changes. All studied strains had more homology with each other in each year and were located in a separated cluster [35]. This proves that isolated strains have not originated from those identified in the previous year in this study period. It is more likely that they were transmitted to the country by travellers or contaminated food sources.

This study shows that a new investigation is required to discover the main source of this outbreak by testing samples from humans and examining the suspected food sources. This kind of investigation needs a close cooperation between different authority divisions, including those exterior to the Health Ministry. There are various reports from other countries performing this kind of national study, that insist on the multiple challenges in implementation of collaborations between involved organizations [34–36]. WHO Advisory Group supports one such programme called 'One Health Projects', which aims to establish and develop programmes that would integrate different projects of antimicrobial resistance surveillance by tracing food-borne pathogens.

PulseNet is an international partnership between different countries that provides access to the analysed images but not to Integrated Surveillance of Antimicrobial Resistance. This programme could be helpful to find out how an outbreak has spread if enough data are provided from all the suspected sources of infection. Multi-national attempts are needed to eradicate cholera infection. First, these programmes are needed because people have to be aware of adequate hygiene measures and provided with safe water and food.

Conclusion

Based on analysed data, all strains collected in the year 2015 showed heterogeneity with strains collected from previous years' outbreaks, although they had some similarities. However, more investigations are required to find out what was the exact origin of the local outbreak that occurred in 2015. Cholera infection could be eradicated in Iran if enough attention were paid to the safety of travellers who arrive in this country.

Conflict of interest

The authors declare that they have no conflict of interest.

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