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Correlation of the Native Inaba Strain With the Dominant Isolated Strains Obtained From Outbreaks in 2013 in Iran

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Abstract

Background: Cholera is an endemic disease in Iran and each year we are faced with some outbreaks throughout the country.

Objectives: The objective of this study was to analyze the isolated cholera strains from outbreak in 2013 and study their similarity and compare their homology in order to find out the route of infection whether emerged from foreign strains or reemerged from domestic native strains.

Materials and Methods: All diagnosed *Vibrio cholerae* isolates were entered to the study after re-identification at referral laboratory of Ministry of Health and Medical Education based on standard procedures. These specimens were examined for specific serogroups by O1 polyvalent and Ogawa/Inaba monospecific antisera and tested by MIC Test Strip Method against ciprofloxacin (CIP), nalidixic acid (NA), cefixime (CFM), ampicillin (AMP), tetracycline (TE), trimethoprim-sulfamethaxazone (SXT), and erythromycin.

Results: A total of 257 clinical *V. cholerae* was isolated from 2013 outbreak in Iran. The dominant causative type was Inaba. *Vibrio cholerae* was reported and isolated from 12 provinces, while 81.71% of cases was from two southeast provinces. The outbreaks started from August and lasted till November. According to antibiotic susceptibility test, isolates were 100% resistant to NA, TE, and SXT, while all were sensitive to CIP, CFM, and AMP. Twenty-three percent of strains were sensitive to erythromycin and all were isolated at the first two weeks of outbreak either from Iranian citizens or from foreign travelers. Homology of isolates was investigated through genotyping by pulse field gel electrophoresis (PFGE) method and their clonality was compared with previously-isolated Iranian native strains. Overall, 92% of analyzed strains showed a homolog pattern. These strains were located in 8 clusters. Although isolated strains in 2011 had 80% homology to recent isolates, they were located in a totally distinct cluster from all strains isolated in 2013. PFGE analysis revealed no dissimilarity between resistant and sensitive stains to erythromycin.

Conclusion: This study confirmed that isolated Inaba strains in 2013 had different clonality patterns in PFGE from previously identified strains, suggesting the presence of a foreign route, namely, from the neighboring countries.

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Background

According to the World Health Organization (WHO) report in 2012, it is estimated that approximately 3–5 million cholera cases occur every year, while only small cases are reported to WHO especially in our regional countries such as Afghanistan, Pakistan, India, and Bangladesh.[1] Based on the WHO data analysis, cholera outbreaks are explained by environmental and climatic factors especially by disasters such as flood and earthquake.[2,3]

Previous studies have indicated that the trend of cholera isolates have been toward Inaba during 2005-2010 outbreaks.[4-6] But the cause of registered outbreaks that spread throughout the whole country of Iran was Ogawa serotype in 2011.[7] The emergence of this serotype was an alarm in Iran after about seven years for import of new *Vibrio cholerae* clones from out of the country. However,

all documents of the Center for Disease Control of Iran shows that it switched to Inaba in 2013, after 2 years. It could have been re-emerged from native reservoir or from strains imported from abroad outbreaks. Based on a previous study, a few Inaba had been isolated from sporadic cases.[7]

Molecular typing techniques such as random amplified polymorphic DNA (RAPD), ribotyping, and multilocus enzyme electrophoresis (MEE) have been employed to study genetic relatedness.[8,9] However, PFGE has been able to play a considerable role in epidemiological investigations because it has a high discriminatory power.[10,11] The pulse field gel electrophoresis (PFGE) protocol for cholera has been validated and standardized. The technique has been accepted to discriminate the data of those participating laboratories and compare their correlations and homology. Therefore it can be applied as a strong tool in monitoring and controlling the enteric pathogen.[12]

PFGE is considered as a "golden standard" molecular typing method for food borne pathogens, illustrating high discriminatory power for epidemiology investigations. This method is able to support epidemiological data in describing how a *V. cholerae* O1 isolate can emerge from abroad or reemerge from native strains. Based on a previous study, 7 pulsotypes were reported from which 3 types were dominant throughout the country and 4 were sporadic,[7] while in previous studies just two types were detected.[5,6]

Objectives

The objective of this study was the analysis of Inaba strains isolated from 2013 outbreak to study the similarity of the isolated strains and compare their homology in order to find out the route of infection whether emerged from foreign strains or reemerged from domestic native strains.

Materials and Methods

All patients suspected for cholera were entered to this study. All *V. cholerae* isolates were diagnosed in the local laboratories of provinces based on standard procedures.[13,14] For re-identification for final confirmation, the first 5 diagnosed *V. cholerae* strains were transferred from each province to the Health Reference Laboratory as a referral laboratory, which is established as a surveillance system by the Ministry of Health and Medical Education.[15,16] These specimens were examined for specific serogroups by O1 polyvalent and Ogawa/Inaba monospecific antisera (BD, Becton Dickinson Co., USA) after identification by standard biochemical and bacteriological tests.

Antimicrobial Susceptibility

Those confirmed *V. cholerae* isolates were tested by MIC Test Strip Method using Liofilchem (CE IVD approved, Italy) against ciprofloxacin (CIP), nalidixic acid (NA), cefixime (CFM), ampicillin (AMP), tetracycline (TE), trimethoprim-sulfamethaxazone (SXT), and erythromycin (E). The organisms *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 29213), and *Pseudomonas aeruginosa* (ATCC 27853) were used as quality control strains for MIC E-test.[17]

Pulse Field Gel Electrophoresis

Genotyping of isolates was performed by PFGE using PulseNet standard procedure for *V. cholera*.[12] The whole agarose-embedded genomic DNA from *V. cholera* was prepared. The conditions used for separation were as follows: an isolated colony was streaked from test cultures to Trypticase soy agar with 5% defibrinated sheep blood (TSA-SB) plates incubated overnight for confluent growth.[7,13] Grown colonies within 14-18 hours were used to prepare cell suspension. Bacterial suspension was prepared in a cell suspension buffer (100 mM Tris:100 mM EDTA, pH 8.0) and adjusted to absorbance values of 0.8-1.0 at a wavelength of 610 nm after which plugs were prepared with SeaKem Gold Agarose (Lonza, Rockland, ME, USA) and proteinase K. Bacterial plugs were lysed (50 mM Tris: 50 mM EDTA, pH 8.0 + 1% sarcosyl, and 25 µL proteinase K 20 mg/mL) and washed with pre-heated sterile ultrapure water and sterile TE buffer 6 times in a 54-55°C water bath. Each plug was digested with 40 units of *Not*I restriction enzyme (Fermentas). DNA molecular weight size marker was prepared by *Xba*I digestion of *Salmonella enterica* serotype Braenderup H9812 plugs. PFGE was carried out with CHEF Mapper XA System (Bio-Rad) using program explained by PulseNet.

Image Analysis

The PFGE fingerprint pattern was analyzed using the computer software package BioNumerics 6.6 (Applied Maths, Belgium). After background subtraction and gel normalization, the fingerprint patterns were subjected to typing on the basis of banding similarity and dissimilarity using Dice similarity coefficient and clustering based on the unweighted-pair group method using average linkages (UPGMA), as recommended by the software manufacturer, and results were graphically represented as dendrograms.

Results

Totally 257 cholera cases were recorded by the authorities throughout the country, in 12 provinces during the outbreaks in 2013. The highest cholera rate (55.25%) was recorded in Baluchestan with 142 cases while other engaged provinces were Kerman, Tehran, Fars, Hormozgan, Qazvin, Qom, Alborz, Golestan, Esfehan, Khorasan Jonubi and Razavi with 73, 10, 8, 8, 5, 3, 2, 2, 2, 1, and 1 cases, respectively. The mean age of the confirmed patients was 25.52 ± 11. The mortality rate was 1.95%. Out of 257 cases, 45 (17.5%) were Iranian while 210 registered cases were Afghan (81.71%), and the rest were Pakistani travelers. The ratio of male patients to female patients was 89.1%.

According to the issued instructions released by Center for Disease Control and Prevention, just 118 specimens were sent for confirmation and 104 out of them were confirmed as cholera cases, including 3 Ogawa and 101 Inaba strains. These three Ogawa strains were not considered in

this comparison as they did not play an important role in the current outbreak.

Antibiotic susceptibility test revealed 100% resistance of Inaba serotypes to NA, TE, and SXT; while all of them were sensitive to CIP, CFM, and AMP. Susceptibility test showed that only 23% that were isolated from both Afghan travelers and Iranian citizens, were sensitive to erythromycin; although all strains showed intermediate pattern from the second month of outbreak. Isolated specimen in 2011 (30-90) had different patterns. It was sensitive to all above antibiotics except to SXT (Table 1).

The genomic DNA of 31 selected strains plus one belonging to the outbreak in 2011 were digested by *Not*I restriction enzyme, creating 17 to 20 fragments, the sizes of which ranged from 20.5 to 668.9 kb. Cluster analysis by dendrogram of the gel images separated the *V. cholerae* biotype strains into some major clusters, although generally all analyzed strains in 2013 showed 92% homology. These strains were located in 8 clusters. Strains isolated in 2011 had a homology less than 80% and were located in a totally distinct cluster from all strains isolated in 2013.

PFGE analysis revealed no correlation between the stains resistant and sensitive to erythromycin (Figure 1), although susceptible strains were seen at first 2 weeks of commencing outbreaks (Table 2).

Table 1. Results of Susceptibility Test

Antimicrobial Agent	Sensitive	Intermediate	Resistant
	2013	2013	2013
CIP	100%	0%	0%
NA	0%	0%	100%
CFM	100%	0%	0%
AMP	100%	0%	0%
TE	0%	0%	100%
SXT	0%	0%	100%
E	23%	77%	0%

Abbreviations: CIP, ciprofloxacin; NA, nalidixic acid; CFM, cefixime; AMP, ampicillin; TE, tetracycline; SXT, trimethoprim-sulfamethaxazone; E, erythromycin.

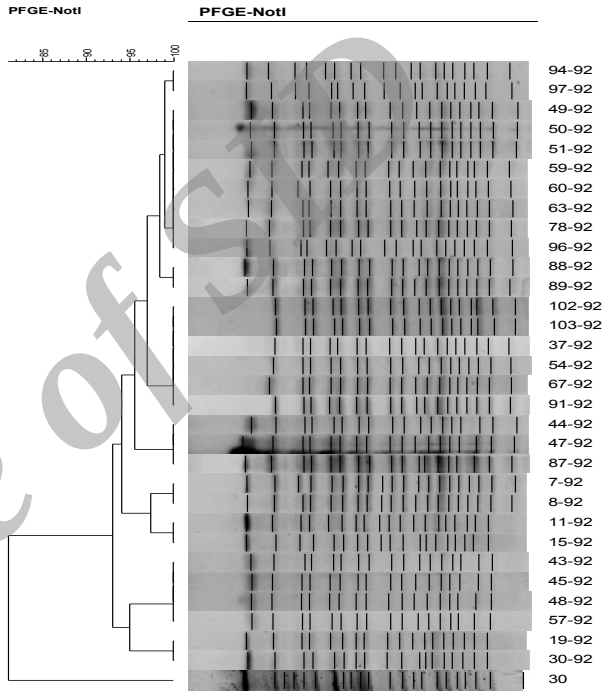

Figure 1. Correlation of PFGE Analysis of Tested Specimens. Abbreviation: PFGE, pulse field gel electrophoresis

Discussion

Mafi et al studied the cholera outbreaks in Iran since 2010 to 2014.[18] They gathered all the data and concluded that the rate of Ogawa strains was reduced from 100% in 2010 to 1.17% in 2013 and rate of Inaba strains was increased to 98.83% in 2013 instead. However, similar studies indicated the trend of isolates toward Inaba during 2005–2010 outbreaks.[5]

Among total cases, 83.65% of all the cases involved with cholera were reported from Baluchestan and Kerman provinces. The Afghan travelers had the major contribution in this cholera outbreak (81.71%). Therefore, it is expected the infection to be transmitted from abroad by the travelers and spread to other provinces from these two provinces like previous outbreaks[18,19]; however, this is needed to be confirmed by a molecular typing method.

Isolated strains were analyzed by the PFGE method using software package BioNumerics for the outbreak in 2011.[7] Results of PFGE analysis in this study revealed the cholera isolates were in different clusters. Ogawa serotype was a main type of the outbreak of 2011. This serotype was distributed through the country with 6 different patterns, while a few Inaba strains were only isolated with differentiated patterns from Ogawa serotype.[7] In the present study, the diversity of cholera strains in 2013 outbreak was studied and the results were compared with the homology of detected Inaba strains in 2011. We concluded the previous native Inaba strain could not have a role in 2013 outbreak.

PFGE results showed no correlation between pattern of our pulsotypes and susceptibility testing results. This result was also supported by different researches.[20,21] The *V. cholerae* strains isolated in this study had nearly similar susceptibility pattern, except to erythromycin. The specimens isolated in 2011 that were located in a separate cluster had different susceptibility patterns, although the Inaba isolated strains with different patterns of susceptibility to erythromycin had no distinct PFGE patterns. However, it seems this issue needs to be more investigated in other researches. Therefore, we suggest that the susceptibility testing be performed for all confirmed isolates during the outbreaks, not just for the first identified strains. It means, we need to revise our previously released sampling procedure.[22,23]

In recent years, new pathogenic variants of *V. cholerae* have emerged and spread throughout many Asian and African countries.[2,24] On the other hand, the emergence of multidrug resistant *V. cholera* isolates has been reported frequently that is a major problem in developing coun-

Table 2. Susceptibility Results of Isolated Strains to Erythromycin

	Code No.	Region	Nationality	Date of Receiving Specimens	Sensitivity to Erythromycin
1	7-92	Bandar Abbas	Afghan	4 Sep	Intermediate
2	8-92	Iranshahr	Afghan	8 Sep	Sensitive
3	11-92	Iranshahr	Afghan	8 Sep	Intermediate
4	15-92	Iranshahr	Afghan	8 Sep	Sensitive
5	19-92	Iranshahr	Afghan	8 Sep	Intermediate
6	30-92	Zahedan	Afghan	9 Sep	Intermediate
7	37-92	Zahedan	Afghan	9 Sep	Intermediate
8	43-92	Qazvin	Afghan	11 Sep	Sensitive
9	44-92	Qom	Iranian	15 Sep	Intermediate
10	45-92	Karaj	Iranian	16 Sep	Intermediate
11	47-92	Gorgan	Iranian	16 Sep	Sensitive
12	48-92	Qazvin	Iranian	17 Sep	Sensitive
13	49-92	Tehran	Afghan	17 Sep	Sensitive
14	50-92	Tehran	Iranian	18 Sep	Intermediate
15	51-92	Jiroft	Iranian	19 Sep	Intermediate
16	54-92	Jiroft	Iranian	19 Sep	Intermediate
17	57-92	Rudbar-e-baluchestan	Afgan	19 Sep	Intermediate
18	59-92	Tehran	Iranian	23 Sep	Intermediate
19	60-92	Qazvin	Afghan	24 Sep	Intermediate
20	63-92	Zahedan	Afghan	24 Sep	Intermediate
21	67-92	Zahedan	Afghan	24 Sep	Intermediate
22	78-92	Zahedan	Afghan	24 Sep	Intermediate
23	87-92	Tehran-Ray	Iranian	30 Sep	Intermediate
24	88-92	Karaj	Iranian	30 Sep	Intermediate
25	89-92	Tehran	Iranian	7 Oct	Intermediate
26	91-92	Bandar Abbas	Iranian	7 Oct	Intermediate
27	94-92	Bandar Abbas	Afghan	14 Oct	Intermediate
28	96-92	Kashan	Afghan	15 Oct	Intermediate
29	97-92	Tehran-Ray	Afghan	15 Oct	Intermediate
30	102-92	Larestan-Fars	Afghan	3 Nov	Intermediate
31	103-92	Bandar Abbas	Afghan	12 Nov	Intermediate

tries, particularly in Iran which is surrounded by several neighboring countries engaging with cholera problem.[25] The number of male cholera patients, their nationality, and the mean age all obviously confirmed the emergence of cholera from abroad. Regarding above mentioned issue, performing an active surveillance program at least during summer for all passengers from Afghanistan and Pakistan is recommended.

Although the main aim of establishment of PulseNet in eastern Mediterranean region is to help the early detection, investigation on bacterial causes of outbreaks could not be traced in the member countries, which causes a gap. It is clear that using recommended standardized procedures for PFGE would help to compare obtained data from different countries.

Conclusion

This study proved that isolated Inaba strains were emerged from neighboring countries, with distinct clonality pattern in PFGE compared to the native strains. The analysis results may support epidemiological data in describing how a *V. cholerae* may distribute through the country. This study also underlined the contribution of new variant of cholera El Tor that had some dissimilarity with the previously isolated strains in outbreak of 2011.

Authors' Contribution

MRF was head of the project; MH was head of Molecular section and corresponding author; MR was head of the Microbiology section and the rest of authors were laboratory staff participated in this research.

Conflict of Interest Disclosures

None.

Ethical Approval

The project was one the main approved duty of Molecular and Microbiology laboratories that confirmed its ethical issue by the the Health Reference Laboratory.

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