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RESEARCH ARTICLE

Evaluating correlation of the native Inaba strain with the dominant isolated strains in outbreaks occurred in Iran at 2013 by Pulsed Field Gel Electrophoresis

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ABSTRACT

Objectives: The aim of this study was to analyze the isolated cholera strains at outbreak 2013 for studying the their similarity and compare their homology in order to find out the route of infection either emerge from abroad or re-emerge from inside native strains.

Methods: All diagnosed *V. cholerae* isolates were entered to the study after re-identification at referral laboratory of Health Ministry based on standard procedures. These specimens were examined for specific serogroups by O1 polyvalent and Ogawa/Inaba nonspecific antisera and tested by MIC Test Strip Method against Ciprofloxacin, Nalidixic Acid, Cefixime, Ampicillin, Tetracycline, Trimethoprim-Sulfamethoxazole, and Erythromycin.

Results: A total of 257 clinical *Vibrio cholerae was* isolated in an outbreak of Iran at 2013. The dominant causative type was Inaba. In Antibiotic susceptibility test isolates were 100% resistant to all except Erythromycin that just 23% of strains were sensitive. Homology of isolates was investigated through genotyping by PFGE method and their clonality was compared with previous isolated Iranian native strain. Overall 92% of analyzed strains showed a homolog pattern. These strains were located in 8 clusters. Although isolated strains at 2011 had 80 % homology with recent isolates, located in complete distinct cluster than all strains isolated at 2013. PFGE analysis revealed no dissimilarity between those stains resistant and sensitive to Erythromycin.

Conclusion: This study confirmed that isolated Inaba strains at 2013 had different clonality pattern in PFGE than previously identified, suggested have foreign route from the neighboring countries. *J Microbiol Infect Dis 2016;6(4): 184-189*

Key words: *Vibrio cholera*, Outbreak, Inaba, PFGE

INTRODUCTION

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

Previous studies indicated the trend of cholera isolates was toward Inaba during 2005-2010 outbreaks [4-6]. But the cause of registered outbreaks that spread throughout the whole country was Ogawa serotype at 2011 [7]. Emergence of this serotype was an alarm in Iran after about seven years for import of new *V. cholerae* clones from out of the country. However all documents of the Center for Disease Control of Iran shown it switched to Inaba after two years at 2013. It could be re-emerging of native reservoir or imported strains from abroad outbreaks. Based on the previous study a few Inaba had been isolated from sporadic cases [7].

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

Pulse-field gel electrophoresis (PFGE) is considered as the ‘‘gold 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 be emerge from abroad or reemerge from native strains. Based on the previous study seven pulsotypes was reported that three type were dominant throughout the country and for sporadic ones [7], while in those previous study just two types had been detected [5,6]. The objective of this study was analysis the isolated Inaba strains at outbreak 2013 to study the similarity of the isolated strains and compare their homology in order to find out the route of infection either emerge from abroad or reemerge from inside native strains.

MATERIALS AND METHODS

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

Antimicrobial susceptibility: Those confirmed *V.cholerae* isolates was 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-Sulfamethoxazole (SXT), and Erythromycin (E). Following organisms were used as quality control strains for MIC E-testing; *E.coli* (ATCC 25922), *S. aureus* (ATCC 29213), and *P. aeruginosa* (ATCC 27853) [17].

PFGE: Genotyping of isolates was performed by pulsed-field gel electrophoresis using Pulse Net 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 by pre-heated sterile ultrapure water and sterile TE buffer six times in a 54-55ºC water bath. Each plug was digested with forty units of *Not* I restriction enzyme (Fermentas). DNA molecular weight size marker was prepared by *Xba* I digestion of *Salmonella enterica* serotype BraenderupH9812 plugs. PFGE was carried out with CHEF Mapper XA System (Bio-Rad) using program explained by Pulse Net.

Image analysis. The fingerprinting pattern in the PFGE gel 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 the Dice similarity coefficient and clustering based on the unweighted-pair group method using average linkages (UPGMA), as recommended by the software manufacturer, and results are graphically represented as dendrograms.

RESULTS

Totally 257 cholera cases were recorded by the authorities throughout the country and just in 12 provinces during the outbreaks occurred in 2013. The Highest Cholera rate (55.25%) were recorded at Baluchistan with 142 cases while other engaged provinces were Kerman, Tehran, Fars, Hormozgan, Qazvin, Qom, Alborz, Golestan, Esfahan, 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 the 210 registered cased were afghan (81.71%), and the rest were Pakistani travelers. The Ratio of male to female and 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 because they had not an important role in the current outbreak.

Antibiotic susceptibility test revealed 100% resistance for Inaba serotypes to Nalidixic acid, Tetracycline and SXT and while all of them were sensitive to Ciprofloxacin, Cefixime and Ampicillin. Susceptibility test showed only 23% were sensitive to Erythromycin that were isolated from both Afghan travelers and Iranian citizen, although all strains were showed intermediated pattern from the second month of outbreak. Isolated specimen at 2011 (30-90), had different pattern. It was sensitive to all above antibiotics except SXT (Table 1).

Table 1. Results of susceptibility test

Antimicrobial Agent	Sensitive	Intermediate	Resistant
	2013	2013	2013
Ciprofloxacin	100%	0%	0%
Nalidixic Acid	0%	0%	100%
Cefixime	100%	0%	0%
Ampicillin	100%	0%	0%
Tetracycline	0%	0%	100%
SXT*	0%	0%	100%
Erythromycin	23%	77%	0%

The genomic DNA of the 31 selected strains plus one belonging to outbreak 2011 was 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 at 2013 showed 92% homology. These strains were located in 8 clusters. Strains isolated at 2011 were also less than 80 % homology and were located in complete distinct cluster than all strains isolated at 2013.

PFGE analysis revealed no correlation between the stains resistant and sensitive to Erythromycin (Figure1), although susceptible strains were seen at first two weeks of commencing outbreaks (Table2).

Table 2. Susceptibility Results to Erythromycin of isolated strains Isolated at this study

	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-Baluchistan	Afghan	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

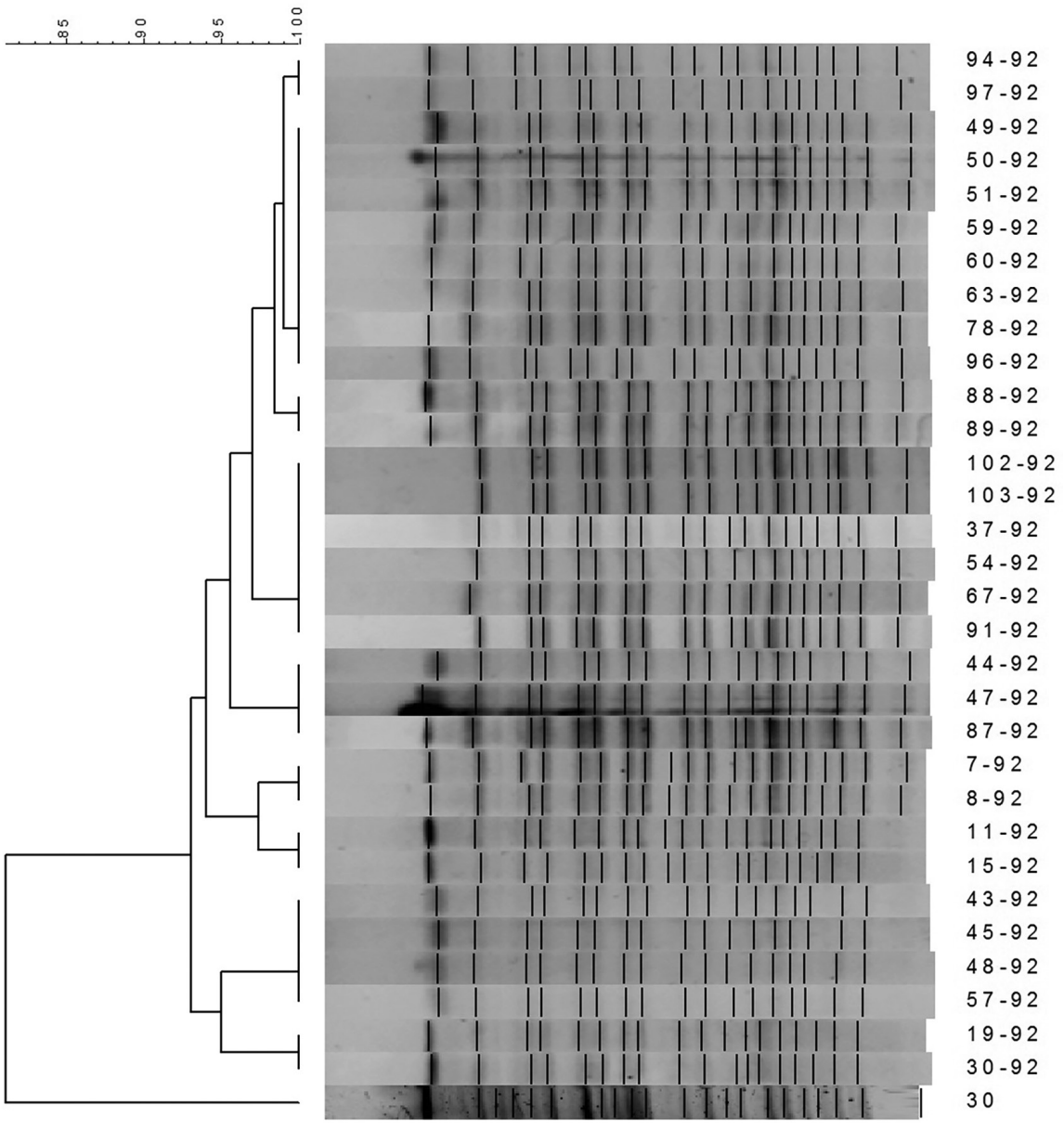

Figure1. Correlation PFGE analysis of Tested Specimens

DISCUSSION

Mafi and his colleagues have studied the cholera outbreaks in Iran since 2010 to 201[18]. They have gathered all the data and concluded that Ogawa strains have reduced from 100 at 2010 to 1.17% at 2013 and rate of Inaba strains have increased to 98.8% in 2013 instead. However, similar studies indicated the trend of isolates toward Inaba during 2005-2010 outbreaks [5].

Among total reported cases 83.65% of the all cases involved with cholera in Baluchistan and Kerman provinces. The Afghan travelers had the majority contribution of the cholera engagement in this out break (81.71%). Therefore, it is expected the infection to be transmitted from abroad by the travelers and spread to other provinces from these two province such as previously outbreaks [18, 19], but needs to be confirmed by a molecular typing method.

Isolated strains have been analyzed by the PFGE method using software package BioNumerics for the outbreak 2011 [7]. Analysis results of PFGE in this study revealed the cholera isolates

were in different clusters. Ogawa serotype was a main type of the outbreak of the 2011. This serotype was distributed through the country with six different patterns, while a few Inaba strains were only isolated with differentiated patterns from Ogawa serotype [7]. At the present study the diversity of cholera strains at the outbreak 2013 was studied and the results were compared with the homology of detected Inaba strains at 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 reported by different researches [20,21]. The *V.cholerae* strains isolated in this study had nearly similar susceptibility pattern, except in Erythromycin. The specimens isolated at 2011 that located in separated cluster had different susceptibility pattern, although those Inaba isolated strains with different result in Erythromycin results had no distinct PFGE patterns. However, it seems this issue needs to be more investigated in separate researches. Therefore, we sagest susceptibility testing should be performed for all confirmed isolates during the outbreaks not just for the first identified strains. It means, we need to revise our previous released sampling procedure.

In recent years, new pathogenic variants of *V. cholerae* have emerged and spread throughout many Asian and African countries [2]. On the other hand emergence of multidrug resistant *V. cholera* isolates has been reported frequently [5] that is a major problem in developing Countries, specially Iran surrounded by several neighboring countries engaging each with cholera problem. The number of male cholera patients, their nationality and the mean age all obviously confirmed emerging cholera from the abroad. Regarding above mentioned point performing an active surveillance program at least during summer season for all passengers from Afghanistan and Pakistan is recommended.

Although the main aim of establishment of Pulse Net in eastern Mediterranean region is to help for early detection, investigation on bacterial causes of outbreaks could not be traced in members causing a gap. It is also needs to mention we could not have access to all specimens therefore this study has performed on the limited number of isolates. It is clear that using recommended standardized procedures for pulsed-field gel electrophoresis (PFGE) would help to compare obtained data from different countries.

CONCLUSION

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

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