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Original article

Investigating the Accessory Colonization Factor (ACF) Cluster in Clinical Strains of *Vibrio cholerae* Isolated during 2011-2012 in Iran

Hajar Mohammadi Barzelighi[1], Marjan Rahnamye Farzami[2], Shabnam Zeighami Alamdari[3], Mohammad Rahbar[4*]

[1] Department of Microbiology, Isfahan University of Medical Sciences, Isfahan, IR Iran
[2] Iranian Reference Health Laboratory, Ministry of Health and Medical Education, Tehran, IR Iran
[3] Food Microbiology Research Centre, Tehran University of Medical Sciences, Tehran, IR Iran
[4] Department of Microbiology, Iranian Reference health Laboratory, Ministry of Health and Medical Education, Tehran, IR Iran

**Corresponding author:* Mohammad Rahbar, Department of Microbiology, Iranian Reference Health Laboratory, Ministry of Health and Medical Education, Tehran, IR Iran, E-mail: Rahbar.reflab@gmail.com, Tel: +982166728112-13, Fax: +982166728121

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Abstract

Background: Accessory colonization factor is located immediately adjacent to and downstream of TCP cluster. These genes (*acf*A, B, C, D) are involved in bacterial colonization and pathogenesis. The aim of this study was to analyze the ACF cluster prevalence rate and gene content in clinical isolates of *Vibrio cholerae*.
Materials and Methods: All of the 21 *V. cholerae* isolates used in this study were collected during 2011-2012 outbreaks in Iran. All of the isolates were screened by biochemical tests and confirmed by specific PCR for 16srRNA-23srRNA intergenic space. The gene content of ACF cluster in the isolates was analyzed using 4 primer pairs with overlapping sequences and then subjected into Restriction Fragment Length Polymorphism (RFLP) assay.
Results: Among the 21 *V. cholerae* isolates, all of them (100%) were identified as *V. cholerae* O1 Inaba, 20 (95%) isolates were determined with El Tor biotype specificity, and 1 isolate (5%) appeared as Classical biotype. A total of 18 strains (85.8 %) contained a complete set of ACF-associated genes, 3 strains (14.2 %) were negative for ACF cluster, and all of the strains showed similar RFLP pattern to each other and to *V. cholerae* ATCC 14035.
Conclusion: The results showed that O1 Inaba was the dominant serotype and positive for ACF cluster in pathogenic *V. cholerae* isolates collected during 2011-2012 in Iran. The presence of some ACF negative strains with potentially pathogenic characteristics proposed that other colonization factors might have been involved in causing pathogenicity and diarrhea in these strains.

Keywords: Accessory colonization factor**,** *Vibrio cholerae***,** Restriction fragment length polymorphism

1. Background

Cholera is a life-threatening form of dehydrating diarrheal disease. Although most of the cholera infections are not detected, but there have been seven recorded pandemics of cholera since 1817(1). Based on the World Health Organization (WHO) annual statistics, *Vibrio cholerae* is responsible for 1.4 to 4.3 million cases of cholera around the world and the occurrence of 28,000 to 142,000 deaths worldwide per year, but this is only the tip of the iceberg since a vast majority of cases are not reported (2). Pathogenic strains isolated from clinic commonly express one of the two O antigens (O1 or O139). The O1 serogroup is categorized into two biotypes, classical and El Tor, which can be differentiate by a number of phenotypic properties. These pathogenic agents are mainly related to the production of cholera toxin (CT), a heat-labile enterotoxin, encoded by the *ctx*AB genes (3-4). Aside from CT, the toxin-coregulated pilus (TCP)(a Type IV pilus) is the other main virulence factor of *V. cholerae*, causing aggregation in *V. cholerae* and inducing microcolony formation within the intestine (5-6).

The *acf* A-D gene cluster encoding accessory colonization factors is physically linked to TCP and under the control of a regulatory cascade directing the synthesis of cholera toxin and other proteins required for colonization (7). The TCP and ACF gene clusters are related to *V. cholerae* chromosome and carried on Vibrio Pathogenicity Island (PAI), a hallmark of epidemic and pandemic *V. cholerae* strains. The function of the various ACF proteins has not been well understood, but it has been found that *acf*A is required for efficient colonization of *V. cholerae* in the intestine as its disruption leads to reduced colonization (14). The *acf*D encodes a lipoprotein (7), *acf*B may interact with *V. cholerae* chemotaxis machinery, and *acf*C is located immediately downstream of *acf*B, all of which are located downstream of the *tcp* genes (8).

2. Objectives

In this research, we attempted to analyze the ACF cluster prevalence rate and to screen their gene content in clinical *V. cholerae* strains isolated from patients during 2011-2012 in Iran using Restriction Fragment Length Polymorphism (RFLP) assay.

3. Materials and Methods

3.1 Bacterial strains

Totally, 21 *V. cholerae* isolates were analyzed in this study. Bacterial strains were previously collected from cholera outbreaks during 2011-2012 in various provinces of Iran (9-10). The specimens were collected by sterile swabs and transported in Carry-Blair medium. The alkaline peptone water and TCBS were used for isolation of the *V. cholerae* strains. Biochemical tests were carried out for identification of the *V. cholerae* isolates, including: oxidase, motility, sucrose and lactose fermentation, growth in 0% NaCl, arginine dihydrolase, ornithine decarboxylase, methyl red, Voges-Proskauer, and indole, as previously indicated (9-10).

The confirmation of biochemical test results were performed by PCR amplification of 16S-23SrRNA intergenic

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space regions of *V. cholerae* genome (10). Serotyping was carried out by polyvalent O1 and O139 and Ogawa and Inaba specific antisera using slide agglutination tests (Mast Diagnostics Ltd., Bootle, Merseyside, UK). The *V. cholerae* ATCC 14035 (O1, Classical biotype, Ogawa serotype) was used as a standard control in all the experiments (11).

3.2. DNA isolation and PCR assay

The genomic DNA was purified using a QIAamp DNA mini kit (Qiagen, USA). PCR used to analyze the presence of ACF element in clinical isolates was accomplished in total volume of 25.5 µL, containing 5 µL template DNA (20 ng), 2.5 µL10XTaq polymerase buffer [100 mM Tris/HCl (pH 8.3), 500 mM KCl, 15 mM MgCl2], 0.25 µL (100 pmolµL^{-1}) of each primer, 0.25 µL (10 mM) dNTPs, 0.2 µL (5 U µL^{-1}) Taq DNA polymerase, and 16.8 µL sterilized distilled water. Four primer pairs used for screening ACF cluster and restriction enzymes are showed in Table 1.

The PCR cycles for identification of isolates were as follow: an initial denaturation at 94ºC for 5 min, 30 cycles of denaturation at 94ºC for 1 min, annealing at 58ºC for 1 min (which was distinctly adjusted for each set of primer pairs), and an extension at 72ºC for 1 min, followed by a final extension step at 72ºC for 3 min.

3.3. RFLP

PCR-RFLP was used to confirm the PCR results and determine the ACF sequence similarity between the isolates. PCR primers and restriction enzymes used in this study are listed in Table 1. RFLP-PCR was carried out in a total volume of 25 µL, containing 2.5 µL 10X buffer specific for each enzyme, 0.6–1 µL restriction enzyme, and 2 µg of each PCR product (12). ACF cluster gene array is depicted in figure 1.

Table 1: Oligonucleotide primers and restriction enzymes used in this study.

Primer designation	Primer sequence (5' →3')	PCR product size (bp)	Restriction Enzyme	Reference
*acf*B-F *acf*C-R	TTGTCTGAGCCGTATGTCG CACTATTTGGGGCAAAAACG	1992	*EcoRV*	21
*acf*1-F *acf*1-R	TCATTGATAGTTTTGGAATC GCTTACAACCTATAAGAAAACCCAAA	2655	*EcoRV*	12
*acf*2-F *acf*2-R	TCATTGATAGTTTTGGGTTTTCTT GCATTGGCTTCAGACTCTCC	2717	*EcoRI*	12
*acf*3-F *acf*3-R	TGGTTTGTATGTACCCCGATA CCAACCTCAATATTAACCCTTAGGA	2121	*EcoRV*	12

4. Results

4.1. Bacterial strains

Bacterial culture in differential media (TCBS and alkaline peptone water) and biochemical tests led to reidentification of *V. cholera* isolates. PCR based identification determined that from a total of 21 *V. cholerae* isolates analyzed in this study, 95% of the isolates were identified as *V. cholerae* O1 El Tor Inaba (isolated in 2011 and 2012), and 5% of the isolates belonged to the classical biotype (only 1 isolate was from 2012).

4.2. Analysis of ACF cluster by PCR-RFLP

Of the 21 clinical *V. cholerae* isolates, 18 strains (85.8 %) contained a complete set of ACF-associated genes, containing bands of 1992, 2655, 2717, and 2121bp for *acf*B-C (encompassing *acf*B and *acf*C), *acf*1F-R (encompassing part of *acf*C and A), *acf*2F-R (encompassing part of *acf*A and D), and *acf*3F-R (encompassing end of *acf*D) primer pairs, respectively (Figure2). Three strains (14.2%) were negative for the whole ACF elements. Digestion of PCR products by *EcoRV*, *EcoRV*, *EcoRI*, and *EcoRV*, respectively, produced bands ranging from 250-1200bp (2 bands ranged ~700 and 1300 for *acf*B-C, 4 bands ranged ~500, 600, 750, and 850 for *acf*1F-R, 4 bands ranged ~ 500, 550, 700, and 1000bp for *acf*2F-R, and 4 bands ranged ~ 250, 450, 600, and 900 for *acf*3F-R). In total, all the strains containing ACF-associated genes, showed similar RFLP pattern to each other and to *V. cholerae* ATCC 14035 (Figure 2).

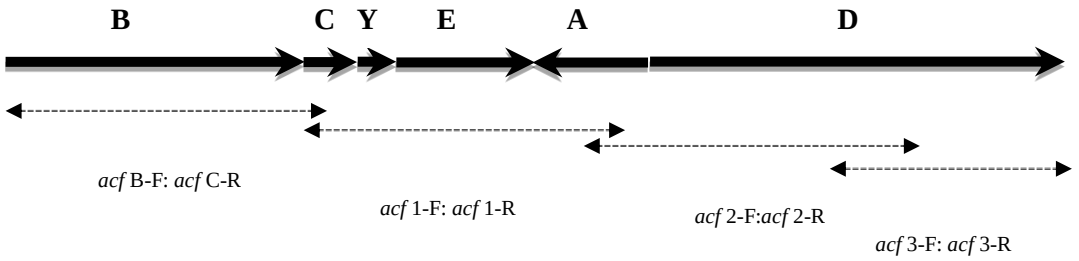

Figure 1. ACF cluster gene array.

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Figure 2.A

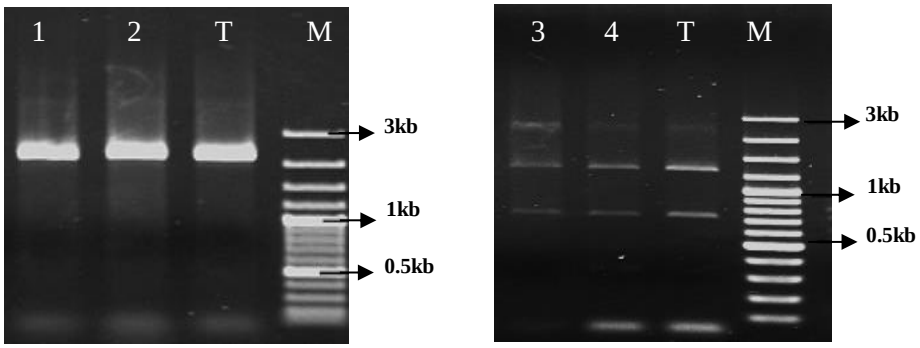

Figure 2.B

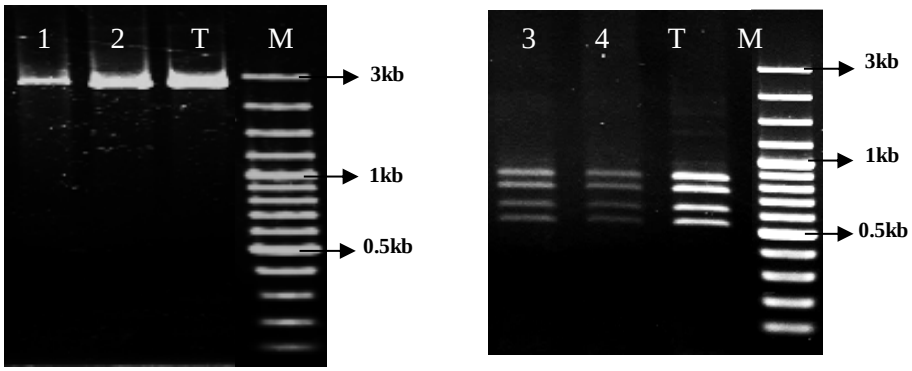

Figure 2.C

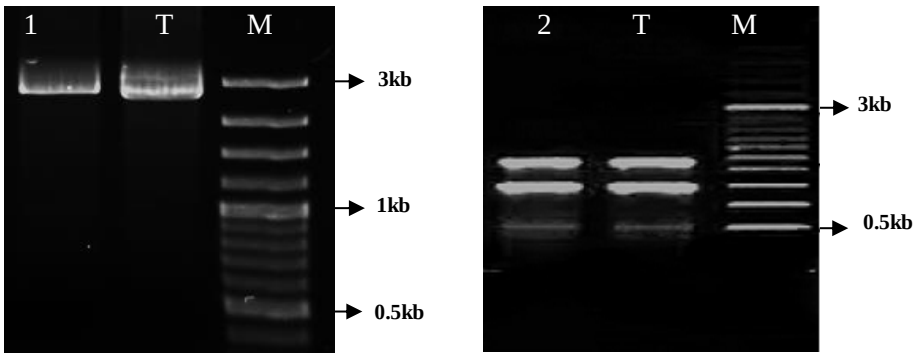

Figure 2.D

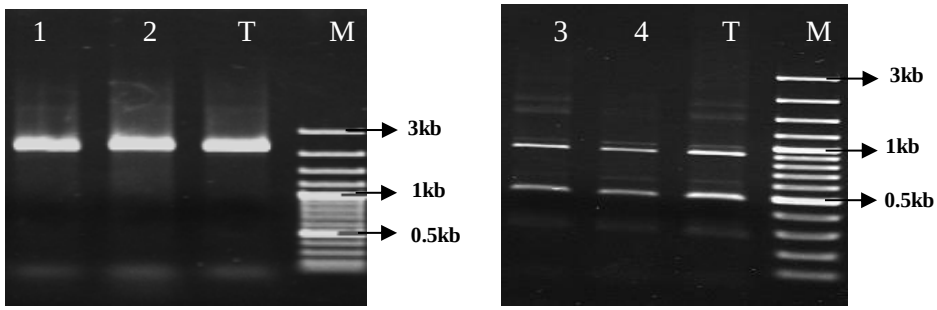

Figure 2: PCR amplification and digestion of A: *acf*B-F: *acf*C-R amplicon by *EcoRV*, B: *acf*1-F: *acf*1-R amplicon by *EcoRV*, C: *acf*2-F: *acf*2-R amplicon by *EcoRI*, and D: *acf*3-F: *acf*3-R amplicon by *EcoRV*. Lanes 1, 2 representatives of *V. cholerae* isolates; laneT *V. cholerae* ATCC 14035; M, 100 bp DNA size marker. Lane 3, 4 digestion of ampilicons by related RE.

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5. Discussion

In the current study, from a total of 21 *V. cholerae* isolates characterized by biochemical and serological tests, 95 and 5% of the isolates were serogrouped as *V. cholerae* O1 El Tor Inaba and *V. cholerae* O1 Classical Inaba, respectively. Most of the outbreaks occurring in Iran during 1977-2005 years were caused by Ogawa serotype. In recent years, a change has been occurred in the statistics of the dominant types of outbreaks in Iran, and there has been a shift between Ogawa and Inaba, as the main types of outbreaks in the country (13). The emergence of classical biotype is considered as a crucial issue because no previous report had been published on the occurrence of classical biotype among the cholera patients in Iran until a study was conducted by Bakhshi et al. in 2014 (9).

ACF cluster is located on the VPI immediately adjacent to and downstream of the TCP cluster and is involved in bacterial colonization and pathogenesis (17). In this study, four primer pairs were used, encompassing the entire ACF cluster structure (*acf*B-D). The *acf*B-C primers covering *acf*B and part of *acf*C were positive in 85.8 % of the isolates; *acf*B encodes an environmental sensor/signal-transducing protein involved in *V. cholera* colonization (15), and *acf*C product activates an ATPase-coupled sulfate transmembrane transporter (16). *Acf*1F-R amplicon containing part of *acf*C, *acf*Y, *acf*E, and a part of *acf*A occurred in 85.8 % of the isolates; *acf*A encodes a protein required for efficient colonization of *V. cholerae* in the intestine (17). *acf*2F-R and *acf*3F-R encompassing segment of *acf*A, *acf*D and distal end of *acf*D, respectively, and similar other amplicons were included in 85.8 % of the isolates. It has been shown that disruption of *acf*D in Ogawa 395 results in decreased cell motility (15). From a total of 21 *V. cholerae* isolates, 18 cases (85.8 %) harbored a complete set of ACF-associated genes (*acf* BCYEAD). The results revealed that dominant *V. cholerae* strains isolated during2011-2012 from Iran possessed intact ACF cluster in their genetic structure. Overall, our results in line with all other published reports indicate the importance of *acf* genes in clinical *V. cholerae* isolates, which are required for enhanced intestinal colonization, signal transduction, passing from transmembrane, and pathogenesis (15-16).

In our study, three clinical isolates which were negative in ACF cluster genes were belonged to El Tor Inaba serogroup. Two isolates of the aforementioned ACF groups were *ctx*$^-$ and *tcp*$^-$, which had been previously indicated by *Bakhshi* et al. in 2014 (9). Another *acf* $^-$isolate in this study was previously characterized as *ctx*+ *tcp*– by *Bakhshi* et al. in 2014 (9). Comparison of these data suggests that probably an excision or recombinational events might have been occurred in CTX and VPI gene cluster (includingTCP and ACF clusters)(18). Basis on these results, we propose that other enterotoxins and colonization factors might have been involved in pathogenic characteristics of these isolates, or such isolates might have acquired different *ctx* or *tcp* alleles (19), and the adaptability of *V. cholerae* as a pathogen might have been facilitated by extensive genetic diversity driven by acquisition and recombination of various genetic elements.

Other colonization factors, including the mannose-fructose-resistant cell associated hemagglutinin (MFRHA) which has been implicated as a virulence determinant and is expressed mostly by the strains of the El Tor biotype also outer membrane proteins (OMPs), have been shown as colonization factors, but their exact roles in colonization is not clear(20).

In continue, RFLP were used to analyze the amplicons sequences and similarities, all the isolates containing ACF cluster (85.7%) displayed the same restriction pattern to each other and to *V. cholerae* ATCC 14035 and probably indicated sequence similarity in ACF structure (*acf*B-D, contain: *acf*BCYEAD) of the isolates. These findings were supported by our previous study, Mohammadi et al. (2011)(12), and Karaolis et al. (2001) (21) studies demonstrating particularly high levels of variation in central segment of VPI (TCP cluster) in comparison to right and left segments.

6. Conclusion

In conclusion, the results obtained in this study include four major points: (i) O1 El Tor Inaba was the dominant serotype in pathogenic *V.cholerae* isolates isolated during 2011-2012, but classical biotype was rarely observed, and this was a concern. (ii)A majority of these dominant *V. cholerae* strains isolated had complete set of ACF cluster in their structure. (iii) The presence of pathogenic potential in some of the ACF-negative *V. cholerae* strains is a reason for thinking about other enterotoxins and colonization factors which might have been involved in *V. cholerae* pathogenicity. (iv) The similarity in digestion pattern probably indicates the sequence similarity in ACF cluster of the isolates and the importance of *acf* genes in *V. cholerae* structure.

Conflict of interests

The authors declare they have no conflict of interest.

Acknowledgments

All of authors contribute to this study

Authors' Contribution

All of authors contribute to this study

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References

1. Umal G, Chandrasekaranl M, Takeda Y, NairM GB. Recent advances in cholera genetics. Curr Sci. 2003;85(11):1538-1545
2. Ali M, Lopez AL, You Y, Kim YE, Sah B, Maskery B, et al. The global burden of cholera. Bull World Health Organ. 2012;90(3):209-18.
3. Finkelstein RA, Feeley JC. Cholera. CRC Crit Rev Microbiol. 1973;2(4):553-623.
4. Mekalanos JJ. Environmental signals controlling expression of virulence determinants in bacteria. J Bacteriol. 1992;174(1):1-7
5. Parsot C, Mekalanos JJ. Structural analysis of the *acf*A and *acf*D genes of *Vibrio cholerae*: effects of DNA topology and transcriptional activators on expression. J Bacteriol. 1992;174(16):5211-8.
6. Meibom KL, Li XB, Nielsen AT, Wu C-Y, Roseman S, Schoolnik GK. The *Vibrio cholerae* chitin utilization program. Proc Natl Acad Sci U S A. 2004;101(8):2524-9.
7. Everiss KD, Hughes KJ, Peterson KM. The accessory colonization factor and toxin-coregulated pilus gene clusters are physically linked on the *Vibrio cholerae* 0395 chromosome. DNA Seq. 1994;5(1):51-5.
8. Brown RC, Taylor RK. Organization of tcp, acf, and *tox*T genes within a ToxT-dependent operon. Mol Microbiol. 1995;16(3):425-39.
9. Bakhshi B, Boustanshenas M, Mahmoudi-aznaveh A. Emergence of *Vibrio cholerae* O1 classical biotype in 2012 in Iran. Lett Appl Microbiol. 2014;58(2):145-9.
10. Bakhshi B. Molecular characterization of *Vibrio cholerae* isolates from Iran 2012 and 2013 outbreaks. Lett Appl Microbiol. 2016;62(6):466-71.
11. Bakhshi B, Mohammadi-Barzelighi H, Hosseini-Aliabad N, Pourshafie MR. Ribotyping and TCP gene cluster analysis of environmental and clinical *Vibrio cholerae* strains isolated in Iran. Ann Microbiol. 2014;64(1):49-53.
12. Mohammadi-Barzelighi H, Bakhshi B, Lari AR, Pourshafie M. Characterization of pathogenicity island prophage in clinical and

[DOR: 20.1001.1.25884107.2017.3.2.3.2]

environmental strains of *Vibrio cholerae*. J Med Microbiol. 2011;60(Pt 12):1742-9.
13. Barati H, Moradi G, Rasouli MA, Mohammadi P. Epidemiologic and drug resistance pattern of *Vibrio cholerae O1*biotype El Tor, serotype Ogawa, in the 2011 Cholera outbreak, in Alborz province, Iran. Jundishapur J Microbiol. 2015; 8(11): e23477.
14. Igbinosa EO, Okoh AI. Toxigenic *Vibrio cholerae* strains and their associated malaises. African J Microbiol Res. 2009;3(5):200-11.
15. Hughes KJ, Everiss KD, Kovach ME, Peterson KM. Sequence analysis of the *Vibrio cholerae**acf*D gene reveals the presence of an overlapping reading frame, *orf*Z, which encodes a protein that shares sequence similarity to the FliA and FliC products of Salmonella. Gene. 1994;146(1):79-82.
16. Heidelberg JF, Eisen JA, Nelson WC, Clayton RA, Gwinn ML, Dodson RJ, et al. DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. Nature. 2000;406(6795):477-83.
17. Hughes KJ, Everiss KD, Kovach ME, Peterson KM. Isolation and characterization of the *Vibrio cholerae**acf*A gene, required for efficient intestinal colonization. Gene. 1995;156(1):59-61.
18. Karaolis DK, Johnson JA, Bailey CC, Boedeker EC, Kaper JB, Reeves PR. A *Vibrio cholerae* pathogenicity island associated with epidemic and pandemic strains. Proc Natl Acad Sci U S A. 1998;95(6):3134-9.
19. Li M, Shimada T, Morris JG, Sulakvelidze A, Sozhamannan S. Evidence for the emergence of non-O1 and non-O139 *Vibrio cholerae* strains with pathogenic potential by exchange of O-antigen biosynthesis regions. Infect Immun. 2002;70(5):2441-53.
20. Jonson G, Holmgren J, Svennerholm A-M. Identification of a mannose-binding pilus on *Vibrio cholerae* El Tor. Microb Pathog. 1991;11(6):433-41.
21. Karaolis DKR, Lan R, Kaper JB, Reeves PR. Comparison of *Vibrio cholerae*pathogenicity islands in sixth and seventh pandemic strains. Infect Immun. 2001;69(3):1947-52.

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