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

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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 ctxAB 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 acfA is required for efficient colonization of *V*. *cholerae* in the intestine as its disruption leads to reduced colonization (14). The acfD encodes a lipoprotein (7), acfB may interact with *V*. *cholerae* chemotaxis machinery, and acfC is located immediately downstream of acfB, 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  $\mu$ L, containing 5  $\mu$ L template DNA (20 ng), 2.5  $\mu$ L10XTaq polymerase buffer [100 mM Tris/HCl (pH 8.3), 500 mM KCl, 15 mM MgCl2], 0.25  $\mu$ L (100 pmol $\mu$ L<sup>-1</sup>) of each primer, 0.25  $\mu$ L (10 mM) dNTPs, 0.2  $\mu$ L (5 U  $\mu$ L<sup>-1</sup>) Taq DNA polymerase, and 16.8  $\mu$ L 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  $94^{\circ}$ C for 5 min, 30 cycles of denaturation at  $94^{\circ}$ C for 1 min, annealing at 58°C for 1 min (which was distinctly adjusted for each set of primer pairs), and an extension at 72°C for 1 min, followed by a final extension step at 72°C 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  $\mu$ L, containing 2.5  $\mu$ L 10X buffer specific for each enzyme, 0.6–1  $\mu$ L restriction enzyme, and 2  $\mu$ g 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 $(5' \rightarrow 3')$	PCR product size (bp)	Restriction Enzyme	Reference
<i>acf</i> B-F <i>acf</i> C-R	TTGTCTGAGCCGTATGTCG CACTATTTGGGGCAAAAACG	1992	EcoRV	21
acf1-F acf1-R	TCATTGATAGTTTTGGAATC GCTTACAACCTATAAGAAAACCCAAA	2655	EcoRV	12
acf2-F acf2-R	TCATTGATAGTTTTGGGTTTTCTT GCATTGGCTTCAGACTCTCC	2717	EcoRI	12
acf3-F acf3-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.



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 amplicons by related RE.

## 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 (acfB-D). The acfB-C primers covering acfB and part of acfC were positive in 85.8 % of the isolates; acfB encodes an environmental sensor/signal-transducing protein involved in V. cholera colonization (15), and acfC product activates an ATPase-coupled sulfate transmembrane transporter (16). Acf1F-R amplicon containing part of acfC, acfY, acfE, and a part of acfA occurred in 85.8 % of the isolates; acfA encodes a protein required for efficient colonization of V. cholerae in the intestine (17). acf2F-R and acf3F-R encompassing segment of acfA, acfD and distal end of acfD, respectively, and similar other amplicons were included in 85.8 % of the isolates. It has been shown that disruption of acfD 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<sup>-</sup> and *tcp*<sup>-</sup>, 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-fructoseresistant 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.

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All of authors contribute to this study

## **Authors' Contribution**

All of authors contribute to this study

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