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Molecular detection of cholera toxin genes in *Vibrio cholerae* infection in human

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ABSTRACT

Cholera is an intestinal infection caused by *Vibrio cholerae*. The hallmark of the disease is profuse secretory diarrhea. Cholera can be endemic, epidemic, or pandemic. A total of 15 *Vibrio cholerae* isolates were obtained from diarrheal samples by standard bacteriological and serological methods. After DNA extraction, a polymerase chain reaction (PCR) assay was performed for detection of the presence of the virulence associated gene such as *ctxAB, hly, ace* and *tcp* genes. In the present study, it was seen that *hly* gene was found in all isolates understudy, whereas *tcp* was detected in 13 isolates of *V. cholerae*. Moreover, ten isolates were shown to possess ace gene in their genome. On the other hand only seven isolates *V. cholerae* were shown to possess *ctxAB* gene. Sequencing of the *tcp* gene from the isolates revealed a novel mutation in the gene. Whereas no mutation found in *ctxAB* and ace genes. *Vibrio cholerae* genome sequence provides a new starting point for the study of this organism's environmental and pathobiological characteristics. It may also provide important clues to understand the metabolic and regulatory networks that link genes on the chromosomes.

KEY WORDS: *Vibrio cholerae*, cholera toxin, PCR, gene sequencing.

1. INTRODUCTION

Cholera remains one crucial problem where in socio-economic conditions commonly fail to encourage sanitary water and other supplies that are essential good for human consumption.

World Health Organization (WHO) show remarkable rise in cholera case since 2006 that never unless, endanger even developing countries with threats of outbreak or /and of epidemic (WHO, 2007). It is most likely that variety of *V. cholerae* serogroups, strains of O1 and O139 among serogroup indeed tie together both the epidemic and pandemic aptitudes (Kumar, 2009).

It is evident that the cholera pathogenesis is in fact, a process that is quite complex besides involving some factors that drive the organism reach to the epithelium of the small intestine and colonize it, producing CT (cholera enterotoxin) which interrupts ion transport by intestinal epithelial cells. Nonetheless, the strains of all pandemic *V. cholerae* retain the *ctxAB* gene on the 6.9-kb CTX prophage that is integrated within chromosome (Ratnam, 2015).

On the other hand, the *V. cholerae* pathogenesis is dependent on the *TCP* (toxin-coregulated pilus) and the *CTX* (cholera toxin) production. Given the fact that it is an adenosine diphosphate ribosylating toxin, the cholera toxin essentially triggers adenylate cyclase in host cells. However, the molecular mechanism of TCP biosynthesis probably involves many of the genes present in the *tcp* gene cluster.

It is generally that *CTX* which is encoded by *ctxAB*, which causes of severe diarrheal symptoms elicited by *V. cholerae*. cholera toxin is consist of one A sub-unit (*ctxA*) that provides the intracellular activity, and five B sub-units (*ctxB*) that binds holo-toxin to the cell receptor (Pal, 2014).

The gene encoding cholera toxin is situated on a 4-5kb DNA segment, flanked by 2 or more copies of a DR (direct repeat) sequence which varies in length from 2.4 to 2.7 kb. The fact that the *ctxAB* gene is present to confirms the toxigenicity of *V. cholerae*.

The pathogenesis of *V. cholerae* essentially rely on the production of the *TCP* & *CTX*, the biogenesis of toxin-coregulated pilus is rely on the *tcp* operon, which consist of a large cluster of 12 genes (Krebs and Ronald, 2011).

The functions of toxin-coregulated pilus are secretion of the colonization factor, and is responsible for the formation micro-colony by mediating the interactions between bacteria that are thought to promote *in vivo* colonization (Krebs and Ronald, 2011).

Moreover, *Ace* (accessory cholera enterotoxin) which considered the 3rd toxin, along with zonula occludens toxin (*Zot*) and cholera toxin (*CT*), which causes the endemic disease cholera (Chatterjee, 2011). The gene that encode the accessory cholera enterotoxin is located proximately next to the cholera toxin gene on a 4.5 kilobase region of *Vibrio cholerae* called the core region or virulence cassette. The accessory cholera enterotoxin was expressed efficiently in the methylotrophic yeast *Pichia pastoris* but not present in *Escherichia coli* (Somarny, 2004).

Furthermore, the hemolysin of *Vibrio cholera* (*hly*), is extracellular membrane damaging proteins with a molecular weight of 65,000, belongs to class of dimorphic proteins that can exist in 2 stable states: an oligomeric integral membrane protein and a water-soluble monomer (Banerjee, 2003).

Hemolysin (*hly*A) is a high frequency found in toxigenic and non-toxigenic strains and it causes diarrhea (Kumar, 2008). So, *hly*A could be used to diagnose pathogenic *Vibrio cholerae* and it plays an essential role in

manifestation of cholera. Also, *HylA* act as pore forming toxin and their ability to lyse target eukaryotic cells by punching holes in the plasma membrane, Honda and Finkelstein (1979), have been shown that purified haemolysin is enterotoxic.

2. MATERIALS AND METHODS

Ethical Approval: An informed agreement was achieved from each patient before their inclusion in the study. For every patient or them follower the procedure had been informed before the samples were collected, making absolutely sure that they understood the procedure that was to be carried out.

Patients: A total 120 stool samples, only 15 isolates of *Vibrio cholerae* were obtained from patients suffering from diarrhea by standard bacteriological methods. The samples were obtained from patients who submitted to Al-Hilla Teaching Hospital, Babylon Governorate, Iraq.

Bacterial Identification: Stools samples should be collected in clean leak-proof containers without disinfectant or detergent residue and with tight-fitting, leak-proof lids. Then the Samples were processed on alkaline peptone water for isolation of *V. cholerae*. After incubation period which last 6 to 8hr., the bacterial colonies were isolated from the alkaline peptone water and then cultured on T.C.B.S agar (Thiosulfate-citrate-bile salts-sucrose agar). The colonies on culture media were proven to be *V. cholerae*. So *V. cholerae* were screened by biochemical tests including motility test, oxidase reaction, KIA (Kligler's iron agar or triple sugar iron agar) test, String test, MR-VP test (methyle red-Voges-Proskauer test) (Tamrakar, 2006) and the serology test was done by using slide agglutination antiserum against *V. cholera*.

Agglutination tests for *V. cholerae* somatic O antigens may be carried out in a petri dish or on a clean glass slide. An inoculating needle or loop, or sterile applicator stick, or tooth pick is used to remove a portion of the growth from the surface of media, Emulsify the growth in a small drop of physiological saline and mix thoroughly by tilting back and forth for about 30 seconds. Examine the suspension carefully to ensure that it is even and does not show clumping due to autoagglutination, add a small drop of antiserum to the suspension. Usually approximately equal volumes of antiserum and growth suspension are mixed Mix the suspension and antiserum well and then tilt slide back and forth to observe for agglutination. If the reaction is positive, very strong clumping will appear within 30 seconds to 1 minute (Difco, USA).

DNA Extraction: DNA from bacteria was extracted according to the genomic DNA purification Kit (Geneaid, U.S.A.).

Detection of cholera toxins genes by PCR: Primers and polymerase chain reaction conditions were used to amplify genes of cholera toxins with polymerase chain reaction are listed in table (1). The primers are includes: *ctxAB, tcp, ace, and hlyA* genes. So, each 25µl of polymerase chain reaction composed of 12.5 µl of master mix, 5µl of DNA extraction in concentration 0.1µg/ml, 2.5 µl of each upstream and downstream primer, and 2.5 µl of free nuclease water. Polymerase chain reaction amplification products were seen by gel electrophoresis on 2% agarose gels for 50 min at 65 V.

Table.1. Primer sequence and polymerase chain reaction conditions

Genes	Primers sequences 5′-3′	Product Size (bp)	PCR conditions	Ref.
ctxAB	F:5'–GCCGGGTTGTGGGAATGCTCCAAG -3' R:5–GCCATACTAATTGCGGCAATCGCATG-3'	536	94 ºC 10min 1x 94 ºC 1 min 59 ºC 1 min 72 ºC 2 min 30X 72ºC 7 min 1 X	(Goel, 2007)
tcp	F:5' – CGT TGG CGG TCA GTC TTG- 3' R:5' – CGG GCT TTC TTC TTG TTC G - 3'	805	94 ºC 10 min 1x 94 ºC 1 min 59 ºC 1 min 72 ºC 2 min 30 X 72ºC 10 min 1x	(Goel, 2007)
ace	F: **5'**TAAGGATGTGCTTATGATGGACACCC-**3'**	309	94ºC 2min 1x 94ºC 1 min 59ºC 1min 72ºC 2min	(Kumar, 2009)

	R:**5'**-CGTGATGAATAAAGATACTCATAGG-**3'**		30 X 72ºC 10min 1x	
hlyA	F-5'GAGCCGGCATTCATCTGAAT-3' R-5'CTCAGCGGGCTAATACGGTTTA-3'	480	94ºC 2min 1x 94ºC 1min 59ºC 1 min 72ºC 2min 30 X 72ºC 10min1x	(Kumar, 2009)

Gene sequencing : Sequences of *tcp, ace and ctxAB* were assured by using sequencing analysis. Then products were collected and sent for sequencing at Macrogen Company, S. Korea.

3. RESULTS

Detection of virulence genes: Four primers were used to investigate the presence of four important genes these are *ctxAB, tcp, ace, and hlyA* genes for 15 *V. cholera* isolated from stool samples. It was found that *hlyA* is present in all isolates, with molecular length in (480 bp) as was shown in Fig. (1-A)

Molecular detection of *tcp* gene was observed in (13) isolates of *V. cholerae* with molecular length in (850 bp) as shown in Figure (1-B).

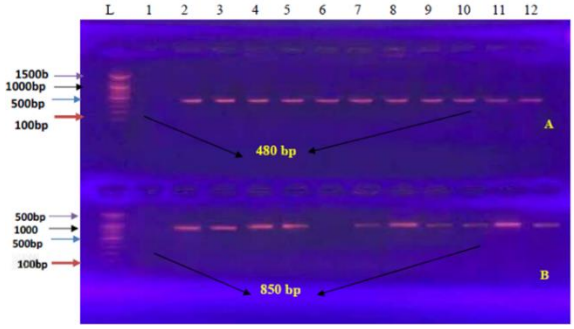


Figure.1. Gel electrophoresis of PCR of A: *hly*, B: *tcp* amplicon; L (ladder) molecular weight marker of ladder (100 bp), 1: negative control, 2:positive control; 3-12 samples obtained from diarrhea patients; agarose 2%.

This study found that (10) isolates of *V. cholerae* gave positive amplicon *ace* gene with molecular length (309 bp) as shown in figure (2-A). It was also found that *ctxAB* marker was observed in (7) isolates of *V. cholerae* with molecular length (536bp) as shown in figure (2-B).

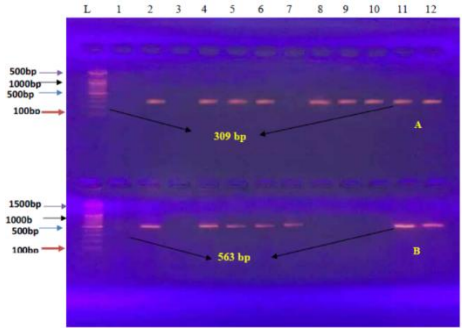


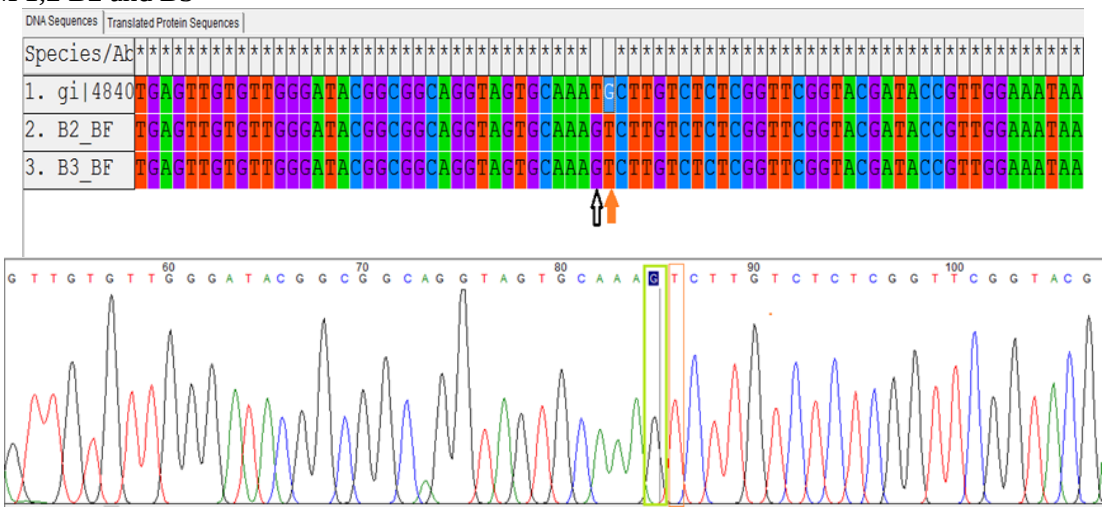
Figure.2. Gel electrophoresis of PCR of A: *ace*, B: *ctxAB* amplicon; L (ladder) molecular weight marker of ladder (100 bp), 1: negative control, 2:positive control; 3-12 samples obtained from diarrhea patients; agarose 2%.

Sequencing of *tcp* gene2: The *tcp* sequence from the selected isolates was amplified by using specific primer and the illation sequences were compared with standard sequence (gi|4840|emb|X64098.1|*Vibrio cholerae tcp* gene cluster for biosynthesis of the TCP pilus. The sequencing achieved at (Macrogen company S. Korea) which use Dye-terminator sequencing method. The sequences results were sent by e-mail as text fasta and waves as PDF files with different colors (red T base, green A base, purple G base and blue C base) as shown in figure.3.

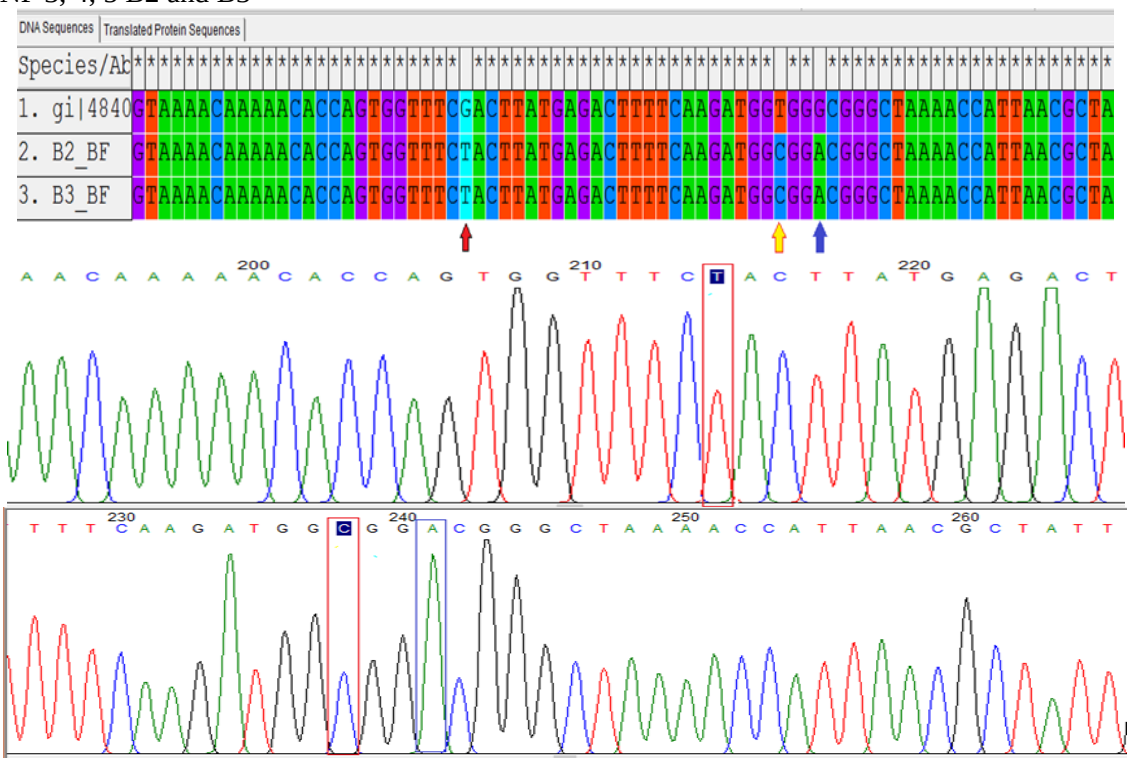
Sequencing of *ace* and *ctxAB* genes: Nucleotide sequences of the *ace* gene for the selected isolates were shown to be identical to standard strain gi|397770463|gb|JX112779.1| *Vibrio cholerae* strain 62013 accessory cholera enterotoxin (ace) gene

Also Nucleotide sequences of the *ctxAB* gene for the selected isolates were shown to be identical to slandered strain gi|48888|emb|X587785.1|:897-1332 *Vibrio* gene for toxin proteins A and B strain 568B.

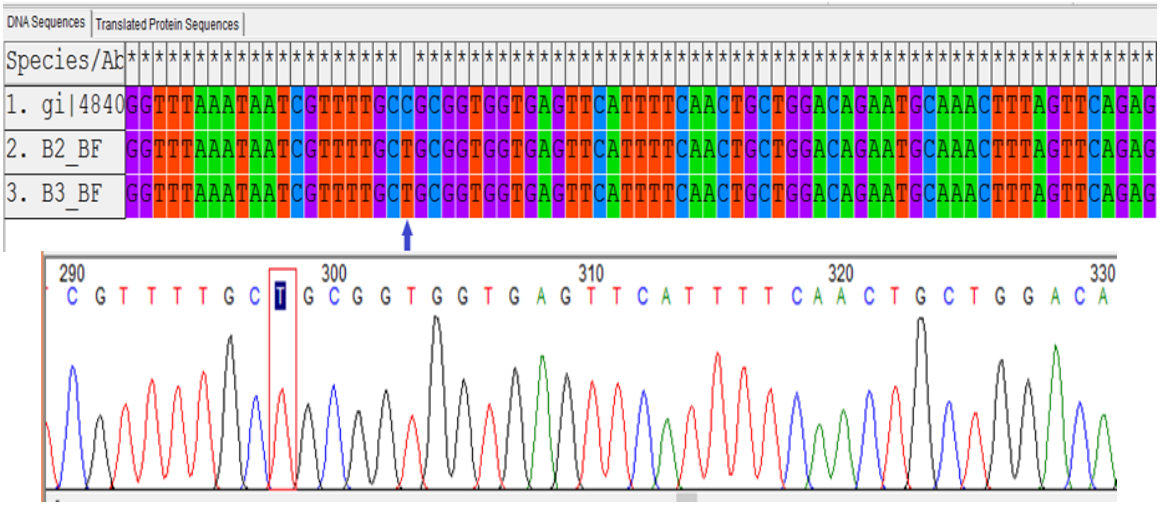
1- SNP1,2 B2 and B3



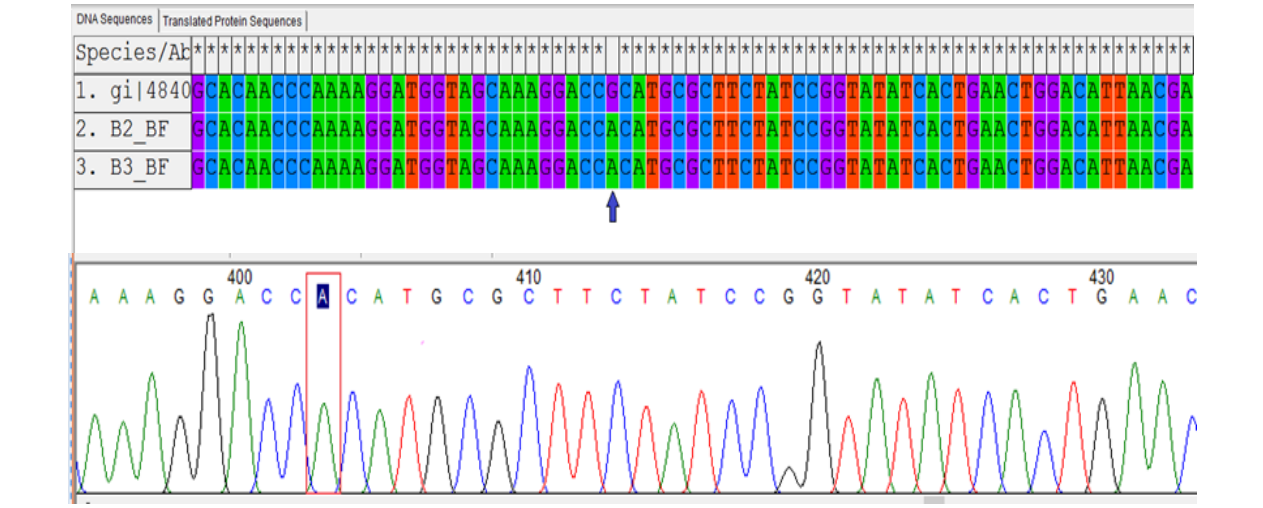
2- SNP 3, 4, 5 B2 and B3



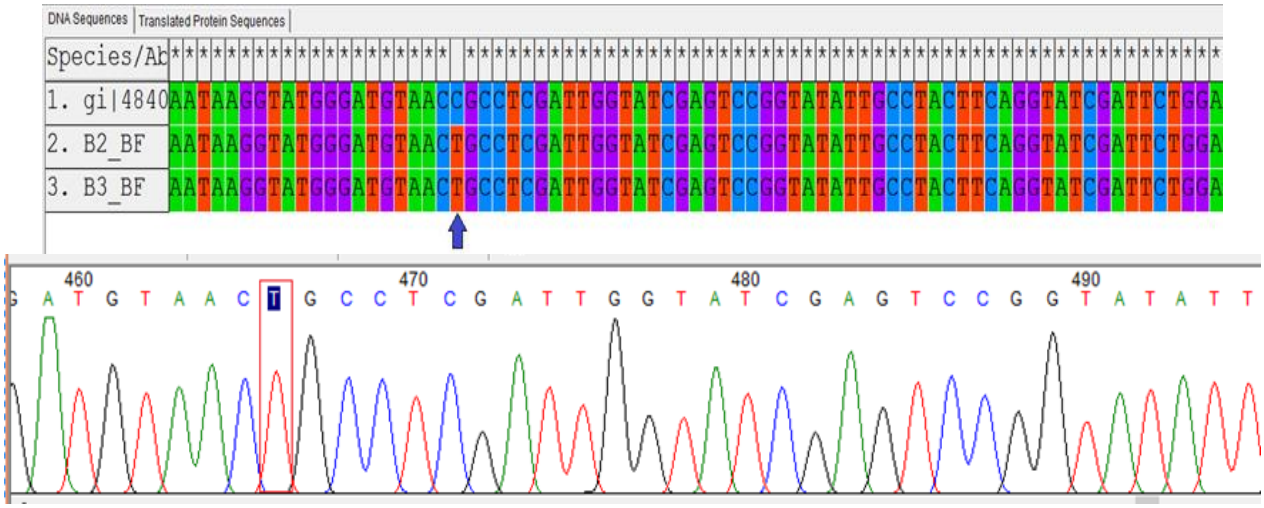
3- SNP 6 B2 and B3



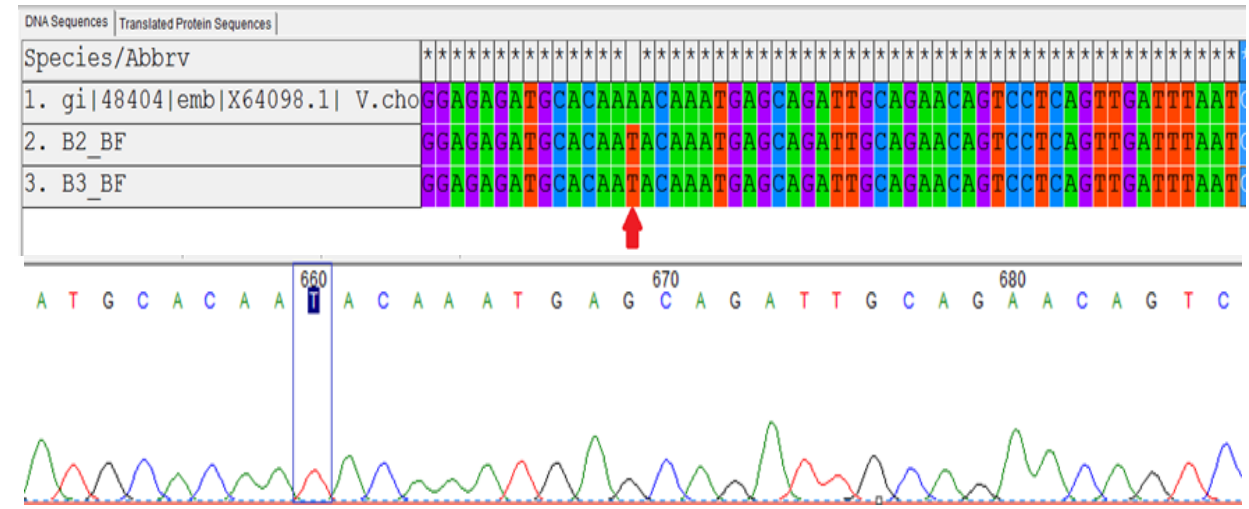
4- SNP 7 B2 and B3



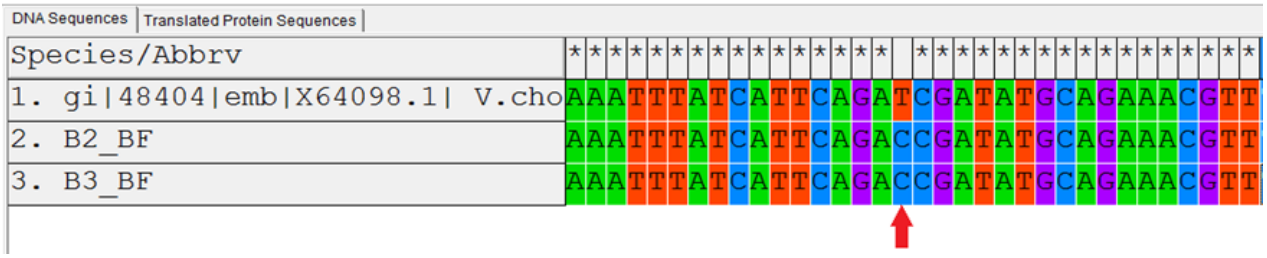
5- SNP 8 B2 and B3

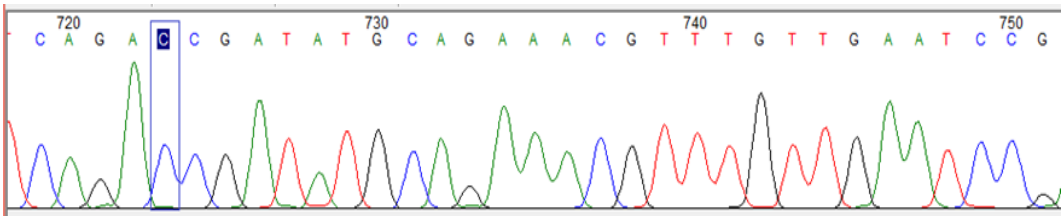


6- SNP 9 B2 and B9



7- SNP 10 B2 and B3





8- SNP 11, 12 B2 and B3

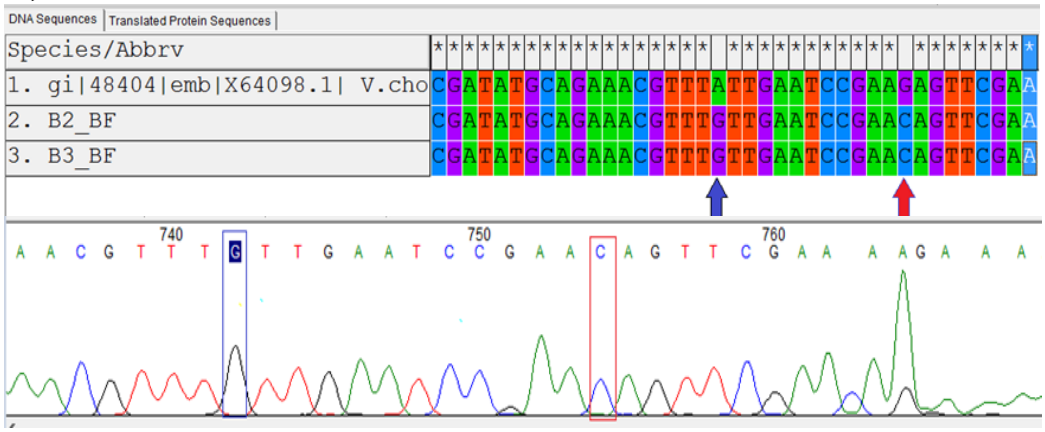


Figure.3. The Fasta and Wave of sequences for *tcp* gene (SNP 1-SNP 12)

DISCUSSION

Detection of gene traits: *Vibrio cholerae* which causes one of the most potent diarrheal diseases in the world. It is expresses virulence factors of different genes such a *hlyA, tcp, ace*, and *ctxAB.*

It was found that *hly*A is present in all isolates at rate (100%). This results was identical with result obtained by Sedaghat, (2013) who detected *hlyA* in (100%) isolates of *V. cholerae*. Hemolysin A gene is considered one of pathogenic factor involved in the cholera disease process, causing releasing a variety of cellular components and host tissue damage (Vidal, 2009). The hemolysin produced is most likely responsible for the enterotoxic activity and is considered to be a virulence factor that causes diarrhea (Alm, 1991).

Goel, (2007) observed that among diverse toxins generated by *V. cholerae*, cholera toxin is the most strong but a number of other genes such as *hly*A and *tcp* are concerned with cholera pathogenesis.

Besides *ctxAB* was present in seven (46.6%) isolates. Radu, (2002) found that this virulence determinant was found at a high rate (96.7%) among diarrhea cases. The *ctxAB* is a coordinated multi-step process that is require the elaboration of a number of virulence factors (Peterson and Mekalanos, 1988). One fundamental step is the successful colonization of the host epithelium by bacteria and this is mediated by their filamentous surface structures such as fimbriae or pili. The cholera toxin is responsible for severe dehydration that results from diarrhea associated with *V. cholerae*. However, the pathogenesis of cholera depend on the synergistic action of a number of other genes and part of the cholera toxin genetic elements comprising *ace* gene, which is capable of causing fluid accumulation in rabbit ileal loops (Fasano, 1991) and *zot* gene, which increases the permeability of the small-intestinal mucosa by affecting the structure of the tight junction (Trucksis, 1993).

However, accessory cholera enterotoxin (*Ace*) gene results is agreement with result obtained by Somarny, (2004) who found the ability of *V. cholerae* to produce *ace* gene at rate (75%). *Ace* which causes milder cholera symptoms. It may contribute to an early phase of intestinal secretion in infections by *V. cholerae*, which can occur prior to the onset of secretion stimulated by the cholera toxin. Also, it is alters ion transport and increases the potential differences across intestinal epithelium Somarny, (2004).

The investigation of toxin-coregulated pilus (*TCP*) gene by PCR technique in 13 isolates was identical with the result obtained by Tomoyuki (2011), who found that (100 %) rate for *tcp*, during the screening of *V. cholerae* isolates. So, *tcp* gene was wanted for pathogenesis of cholera. Colonization of the large bowel and cecum by *TCPV. cholerae* cells might contribute to the shedding of *V. cholerae* by asymptomatic carriers and could play a role in the dissemination of this organism.

Study conducted by Faruque and Nair (2003), has been shown that the major virulence genes of *V. cholerae* which required for pathogenesis in animal models and humans are the genes involved in the production of *TCP* and *CT*.

The ability of the pathogenic strain to cause cholera in humans depends chiefly on two factors: the bacteria has ability to produce toxins, primarily the *CT*, and the ability of bacteria to colonize the host's intestinal epithelium which is mediated by the bacterial adhesion by *TCP*. The genes for the biosynthesis of toxin co-regulated pili are part of the *TCP* pathogenicity island of *V. cholerae* chromosome and involve at least fifteen open reading frames

(Kovach, 1996). The role of toxin coregulated pili as an essential for colonization factor inside the host intestine is well recognized (Hase and Mekalanos, 1998).

However, the isolates were positive *ctxAB, ace, tcp,* and *hlyA* amplicons, this indicating that the isolates containing the virulence gene cassette and the genes encoding surface organelles required for intestinal adherence and colonization were intact.

Singh, (2001) have been documented that *V. cholerae* strain devoid of the virulence gene cassette which cause diarrhea by a mechanism differ from that of cholera toxin-producing *V. cholerae.*

Virulence genotype of *V. cholerae* that are associated with infection may differ according to climatic changes. Some studies showed seasonal patterns of *V. cholerae* transmission. High number of these transmissions occurred in summer compared to winter. Consumption of untreated uncooked seafood and water in summer is epidemiologic evidence of *Vibrio cholerae* transmission (Schirmeister, 2014).

Sequencing of *tcp, ctxAB, and ace*: The results of DNA sequencing should be first examined to confirm the nucleotide sequences and close correlation with others world strains, it has been found that *TCP* gene sequence data revealed 12 single nucleotides polymorphism in region between nucleotide no. 60 to 760 based as shown in result which may cause silent mutation or frame shift mutation.

In accordance with observation by Sheikh (2012), it has been found 97% homologs' between the *tcp* gene in the isolates and stander strain that agree with the result obtained of this study.

Whereas the ace gene and *ctx* gene did not show any types of polymorphisms this due to there are no variation in the isolates of this study with stander at nucleotide level.

It is mostly found that at genus level, sequence based identification could have resulted in similar therapeutic decisions in antibiotic therapy in accordance with species level, given the fact that local resistance surveillance data are available. The genus level identifications are often plausible for estimation of the etiological relevance (Frickmann, 2015).

Within a certain genes sequence difference between related organisms are most likely the results of slow, continual acquisition of random mutations, which are subject to selection and inherited vertically. Differences seen in whole-genome comparisons, however, are the sum of this vertical inheritance and any number of horizontal gene transfer events that involve simultaneous acquisition of many genes through the methods of gene transfer (Zeigler, 2003).

4. CONCLUSION

The *Vibrio cholerae* genome sequence provides a new starting point for the study of this organism's environmental and pathobiological characteristics. It will be interesting to determine the gene expression patterns that are unique to its survival and replication during human infection as well as in the environment, The genome sequence may also provide important clues to understand the metabolic and regulatory networks that link genes on the chromosomes.

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