JOURNAL OF PURE AND APPLIED MICROBIOLOGY, March 2012. Vol. **6**(1), p. 97-102

Multiplex PCR for Identification of *Vibrio cholerae* Genes

Abbas Maleki[1], Sobhan Ghafourian[1], Nourkhoda Sadeghifard[1]*, Iraj Pakzad[1], Reza Mohebi[1], Shiva Hosseini[1], Afra Khosravi[1] and Afshar Alihosseini[2]

[1]Clinical Microbiology Research Center, Ilam University of Medical Sciences, Ilam, Iran.
[2]Department of Chemical Engineering, Ilam Branch, Islamic Azad University, Ilam, Iran.

(Received: 18 February 2011; accepted: 21 April 2011)

Cholera has re-emerged as a major infectious disease in the recent past, with a global increase in its incidence. This study was focused on Prevalence of ace, tcpA, zot and ctxA genes in *Vibrio cholerae* strains associated cholera epidemic in 2005, Iran. Number of Thirty-nine strains of Vibrio cholerae related to cholera epidemic obtained from different provinces in 2005 in Iran. All the isolates identified by using standard bacteriological methods. Multiplex Polymerase Chain Reaction performed for identification of ctxa,tcpa,ace,zot. All the isolates were inaba O1, Of thirty nine inaba 100% were positive for ace and zot,89.74% presented ctxa and 84.61% showed tcpA. It was found that zot and ace genes in all strains, ctxA genes was found in 89/74 per cent and tcpA genes in 84.6 per cent of strains. It has been suggested that the control of cholera epidemics is too big a task for a national diarrheal diseases control programmed. Although this may be true in the case of extensive epidemics, it should not be forgotten that large outbreaks of this kind are often the result of initial delays in detection and containment. A properly organized national control programmed provides the framework for quick detection and prompt containment and is the best means of ensuring preparedness for cholera control.

Keywords: *Vibrio cholera,* Multiplex PCR, Toxigenic, Iran.

The genus *Vibrio* is a highly diverse group of gram-negative bacteria that contains approximately 72 species (www.bacterio .net). The group includes symbionts and commensals that are found in or on marine animals, as well as many species that are pathogenic to animals (Thompson *et.al.*, 2004). There are 12 species that are routinely isolated from human clinical samples, and the diseases in which they are implicated include diarrheal disease, septicemia, and wound infections (Farmer *et.al*., 2003).

Three species account for the majority of human *Vibrio* infections. Toxigenic *Vibrio cholerae* is the causative agent of the disease cholera and is acquired through ingestion of contaminated food or water. Infection may lead to a profuse, watery diarrhea that can lead to severe dehydration and death if left untreated. Cholera has re-emerged as a major infectious disease in the recent past, with a global increase in its incidence. In 1994 cholera cases were notified from 94 countries, the highest ever number of countries in one year (World Health Organization 1995). Two particularly disturbing aspects of the global cholera picture in the 1990s have been the dramatic and unexpected reappearance in January 1991 of epidemic cholera caused by *V. cholera* O1 El Tor in Latin America after a 100-year absence from the region (Tauxe *et.al.*, 1992) and the unprecedented appearance in late 1992 in southern India of an epidemic strain of *V. cholera* non-O1, classified as *V. cholera* O139 Bengal (Ramamurthy 1993). The reasons for these

* To whom all correspondence should be addressed.
E-mail: Sadeghifard@gmail.com

phenomena are still being intensively researched. This study was focused on Prevalence of ace, tcpA, zot and ctxA genes in *Vibrio cholerae* strains associated cholera epidemic in 2005, Iran.

MATERIAL AND METHODS

Bacterial strains

Number of Thirty-nine strains of *Vibrio cholerae* related to cholera epidemic in 2005 from different provinces, including two strains from Tehran (Luqman hospital), eleven strains of Golestan University of Medical Sciences, Zabol a strain, nine strains of Qom Health Center, Four strains of Zahedan Health Center and Twelve strains of Tehran (Emam Khomeini Hospital) were obtained.

Detection of vibrio cholera

All isolates identified by using standard bacteriological methods. All isolates were examined for their oxidase reaction, and the identities of the *V. cholerae* O1 strains were confirmed by serogrouping using growth from triple-sugar iron agar slants with polyclonal O1 and monospecific Inaba and Ogawa anti sera (World Health Organization, 1987).

DNA extraction

V. cholerae strains were cultured in LB broth at 37°C overnight and DNA was extracted according to the published method of Johansson and Woodford.

PCR

PCR primers were: CTXA: F: 5 / – CTC AGA CGG GAT TTG TTA GGC ACG – 3 R: 5 – TCT ATC CTC GTA GCC CCT ATT AAC G-3,TcpA: F: 5 /- CAC GAT AAG AAA ACC GGT CAA GAG – 3, R: 5/ – CGA AAG CAC CTT CTT TCA CGT TG – 3, Ace:F: 5/-AGA GCG CTG CAT TTA TCC TTA TTG -3/, R: 5/-AAC TCG GTC TCG GCC TCT CGT ATC-3, Zot: F: 5/-TCG CTT AAC GAT GGC GCG TTT T-3, R: 5/-AAC CCC GTT TCA CTT CTA CCC A-3.

The size of amplicon for ctxa,tcpa,ace,zot were 301bp,451bp,600bp,947bp ,respectively (Keasler & hall, 1993) (Leal *et al*,. 2004).

PCR carried out for zot¡ tcpA , ctxA under following condition

Initially denaturation at 94°C for 5 min, following by35 cycles of denaturation 95c for 30 second anneling 52°C for 1 min, and 72°C for 1 min, with a final extension of 72°C for 10 min. but for ace annealing temperature were 55c. The amplicons were run in 1% agarose gel. The gels were stained with ethidium bromide and a band observed at desired position was photographed on an ultraviolet light transilluminator

Multiplex PCR

Multiplex PCR had done for zot¡ tcpA , ctxA ,ace genes under following condition, Initially denaturation at 94°C for 5 min, following by35 cycles of denaturation 95c for 30 second annealing 53.5°C for 1 min, and 72°C for 1 min, with a final extension of 72°C for 10 min the amplicons were

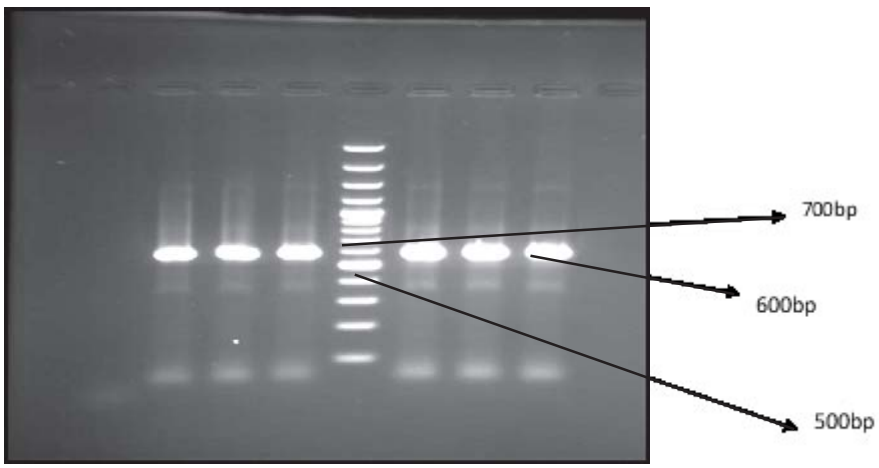

Fig. 1. Ace gene with 600bp molecular weight

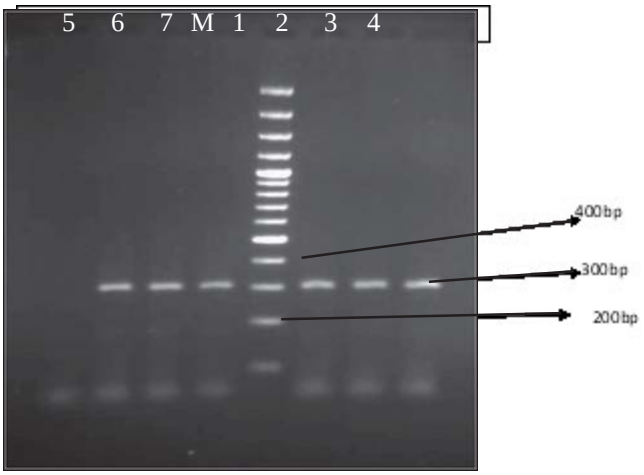

Fig. 2. ctxA gene with 301bp molecular weight

run in 1% agarose gel. The gels were stained with ethidium bromide and a band observed at desired position was photographed on an ultraviolet light transilluminator.

RESULTS

All isolates from patients identified as V. cholerae using standard biochemical tests. All the isolates were determined as a O1 serotype Inaba by serotyping. The results reflected all the isolates were positive for ace gene, while thirty four strains possess ctxA and five *vibrio cholera* were negative for ctxA (Fig. 1,2).

Results of PCR reactions for tcpA gene showed that of thirty-nine strains studied thirty-three strains with tcpA gene existed while six other strains were negative. (Fig. 3).

Results of PCR reactions for zot gene showed that zot observed in all the strains. (Fig. 4).

Multiplex PCR results also administrated that of the thirty-nine inaba isolates, 100 %(n=39) were positive for ace and zot, 89.74 % (n=35) carried ctxa and 84.61% (n=33) had tcpA. Five isolates were positive for ctxA while were negative for tcpA (Fig. 5).

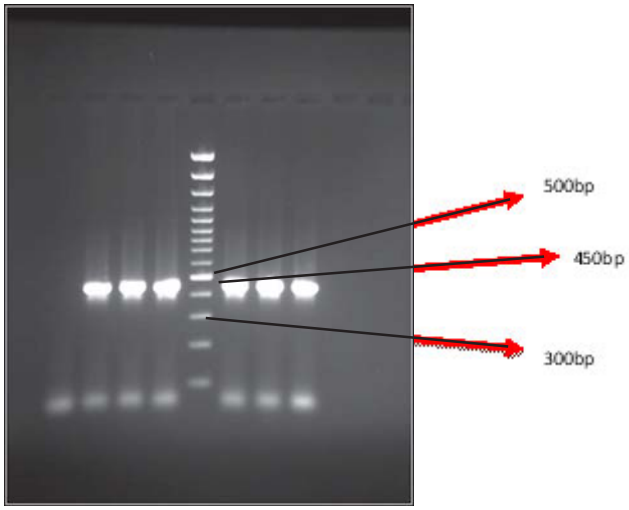

Fig. 3. tcpA gene with 451p molecular weight

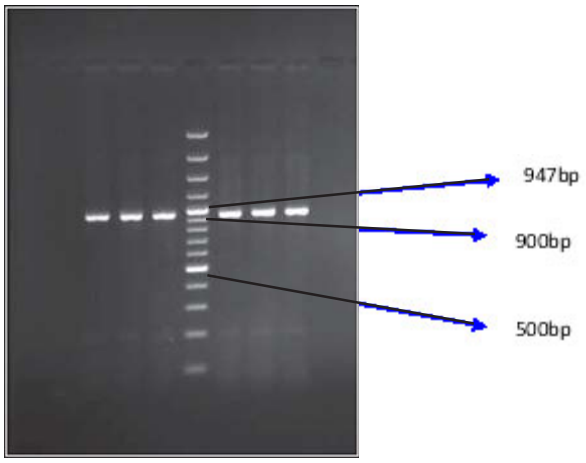

Fig. 4. zot gene with 947bp molecular weight

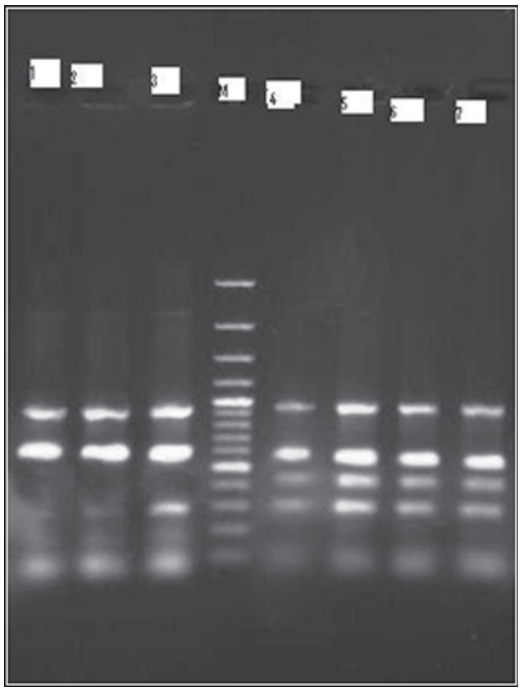

Fig. 5. MULTIPLEX PCR: 1= zot , ctxA 2= zot , ctxA 3= zot ¡ tcpA , **ctxA** M=marker(100bp) 4,5,6,7= ace ¡ zot ¡ tcpA , ctxA

DISCUSSION

Vibrio cholerae is a gram-negative bacterium that cause cholera. Cholera disease of poor communities and human is the only natural host of cholera, the main focus of this disease in India's Ganges River Delta is usually there, or the center of Mecca. The transmission of cholera and the emergence of the epidemic primarily contaminated water and uncooked food, especially seafood and vegetables have an important role. Those microbes and vectors to form with no clinical

signs are of great importance are transmitted (Faruque *et al.,* 1998).

Productions of cholera toxin is caused watery diarrhea, such as water are brass. cholera toxin is coded by the ctxA and ctxB genes. Zot toxin on intestinal tight joints and affects permeability is increased. Sub cholera enterotoxin (ace), potential difference across the intestinal epithelium increases and changes in ion transport. Pathogenic Vibrio cholera depended a set of factors such as the ability to produce toxin, sticking to the small intestine. Through a poster called colonization factor elephantine is done, depends. Expression is elephantine (TCP) jointly with the toxin is done. Cholera toxin and TCP exclusively in clinical strains of *Vibrio cholerae* O1 and O139 can be seen (Ashrafus *et al.*, 2006).

The first study in 1981 that would have many questions about the environmental strains isolated in non-endemic countries that failed cholera toxin presence detect with conventional approaches normal, they respond. May be minor mutations in these strains the genes that code cholera toxin they created and therefore these changes may be reversible and again these environmental strains cholera toxin-producing strains become public health in the community jeopardizing.

PCR technique detection and finding ctx sequences has been used. Sequences of ctx genes among completely *Vibrio cholerae* strains has been maintained. So the sequence variation that may be connected to reduce on the primer oligo ctx gene is not a problem (Goldberg *et al.*,1986).

Leal *et al* in Brazil by using RAPD-PCR technique studied *Vibrio cholerae* O1 strains genes and the presence of ace, zot, tcpA and ctxA in these strains by PCR. Results for virulence genes identified by PCR that all strains of the studied harbored four genes (Leal *et al*,. 2004) .

In this study, PCR on *Vibrio cholerae* O1 serotype strains isolated from epidemic Inaba in Iran to investigate the presence or absence of genes zot, ace, tcpA and ctxA, it was found that zot and ace genes in all strains, ctxA genes was found in 89/74 per cent and tcpA genes in 84.6 per cent of strains. In research had done by Shahcheraghi et al in Qazvin,all isolates were identifiedas *V. cholera* O1, also our results revealed all isolates were *V. cholera* O1 (Shahcheraghi *et al.,* 2009). It has been suggested that the control of cholera epidemics is too big a task for a national diarrheal diseases control programmed. Although this may be true in the case of extensive epidemics, it should not be forgotten that large outbreaks of this kind are often the result of initial delays in detection and containment. A properly organized national control programmed provides the framework for quick detection and prompt containment and is the best means of ensuring preparedness for cholera control.

REFERENCES

1. Ashrafus, S., Bhuyian N., Suraia N., Ansaruzzaman, M., Alam, M., Hamabata, T., Takeda, Y., David, A., Sack A., Balakrish, N.G. Genetic characteristics of Matlab variants of *Vibrio cholerae* O1 that are hybrids between classical and El Tor biotypes. *Med Microbiol.*, 2006; **55,** 1563-1569.
2. Farmer, J. J. III, J. Janda, M., Birkhead, K. *Vibrio*, p. 706–718. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), Manual of clinical microbiology, 8th ed., 2003; Vol. 1. ASM Press, Washington, DC.
3. Faruque, S.M., Albert, M.J., Mekalanos, J.J. Epidemiology, genetics, and ecology of toxigenic Vibrio cholerae. *Microbiol Mol Biol Rev*. 1998; **62**(4): 1301-14.
4. Goldberg, I, Mekalanos, J.J. Effect of a recA mutation on cholera toxin gene amplification and deletion events. *J Bacteriol.,* 1986; **165**(3): 723-31.
5. KEASLER, S.P., Hall, R.H. Detecting and biotyping *Vibrio cholerae* O1 withmultiplex polymerase chain reaction. *Lancet*, 1993; **341:** 1661.
6. Leal, N.C. , Sobreria, M. , Leal-Balbino, T.C. Evaluation of RAPDbasedtyping scheme in a molecular epidemiology study of *Vibrio cholerae* O1, *Brazil. J. appl. Microbiol*, 2004; **96:** 447-454.
7. Ramamurthy, T.,Garg, S, Sharma, R, Bhattacharya, S.K., Nair, G.B., Shimada, T., Takeda, T., Karasawa, T., Kurazano, H., Pal, A. Emergence of a novel strain of *Vibrio cholera* with epidemicpotential in southern and eastern India. *Lancet*. 1993; **341**(8846): 703-4.
8. Shahcheraghi, F., Rhabar, M., Zahraei, S.M., Nikbin, V.S., Shooraj, F. Transmission of *V. cholera* O1seroptype inaba in a rural area in gazvin, Iran associated with Drinking water. *Asian journal of epidemiology.* 2009; **2**(3):

66-71.
9. Thompson, F. L., T. Iida, and J. Swings. Biodiversity of vibrios. *Mol. Biol. Rev.* 2004; **68:** 403–431.
10. Tauxe, R.V., Blake, P.A. Epidemic cholera in Latin America. *Journal of the American Medical Association*, 1992; **267:** 1388–1390.
11. World Health Organization. Program for control of diarrhoeal diseases. In Manual for Laboratory Investigation of Acute Infection, 1987; CDD/ 83.3. Geneva: World Health Organization.
12. World Health Organization. Cholera in 1994. *Weekly Epidemiological Record*, 1995; **70:** 201–208.