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Genomic Fingerprinting of the Vaccine Strain of Clostridium Tetani by Restriction Fragment Length Polymorphism Technique

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Abstract

***Background*:** *Clostridium tetani* or Nicolaier's bacillus is an obligatory anaerobic, Gram-positive, movable with terminal or sub terminal spore. The chromosome of *C. tetani* contains 2,799,250 bp with a G+C content of 28.6%. The aim of this study was identification and genomic fingerprinting of the vaccine strain of *C. tetani*.

***Materials and Methods*:** The vaccine strain of *C. tetani* was provided by Razi Vaccine and Serum Research Institute. The seeds were inoculated into Columbia blood agar and grown for 72 h and transferred to the thioglycolate broth medium for further 36 h culturing. The cultures were incubated at 35ºC in anaerobic conditions. DNA extraction with phenol/ chloroform method was performed. After extraction, the consistency of DNA was assayed. Next, the vaccine strain was digested using pvuII enzyme and incubated at 37ºC for overnight. The digested DNA was gel-electrophoresed by 1% agarose for a short time. Then, the gel was studied with Gel Doc system and transferred to Hybond N+membrane using standard DNA blotting techniques.

***Results*:** The vaccine strain of C. tetani genome was fingerprinted by RFLP technique. Our preliminary results showed no divergence exists in the vaccine strain used for the production tetanus toxoid during the periods of 1990-2011.

***Conclusion*:** Observation suggests that there is lack of significant changes in RFLP genomic fingerprinting profile of the vaccine strain. Therefore, this strain did not lose its efficiency in tetanus vaccine production. RFLP analysis is worthwhile in investigating the nature of the vaccine strain *C. tetani*.

Introduction

Tetanus is a frequently fatal disease caused by the gram-positive and anaerobic bacterium Clostridium tetani. This spore is extremely resistant to physical and chemical agents and also antiseptic material. This bacterium is negative oxidase and catalase. Also, nitrate reduction reactions, indole, methylene blue assay MR (Methil-Red) and Vogef-Proskauer test (VP) is negative for *Clostridium tetani* [1].

Tetanus is not infectious disease but in fact is a preventable vaccine. After entering bacteria to the body, spores germinate and make an exotoxin released by lymphatic and circulatory system [2].

This exotoxin is called tetanospasmin and produced by tetX gen that located in *C. tetani* plasmid [3, 4]. For tetanus vaccine producing, bacteria toxin is inactive. Infact, the tetanus toxoid, has immune stimulating properties. Tetanospasmin is a neurotoxin because binds to the inhibitory nerve receptors in the central nervous system and inhibits release of neurotransmitters. As a result, the resistance in muscles contraction is reduced. This means that the muscles remain contracted. The patient may have symptoms of muscle spasms and difficulty to swallowing [2]. Therefore, studies of genomic DNA in Clostridium tetani is very important. A reliable molecular technique to perform such studies is RFLP (Restriction Fragment Length Polymorphism). The study was conducted for the first time in Iran and world. Molecular identification and genomic fingerprinting of vaccinal *C. tetani* (Harvard strains) was carried out by RFLP. The aim of this research is to study any genetic change or mutation in the bacteria genome of Harvard *C. tetani* by designed molecular probes. Therefore, vaccinal strains that using to produce toxoid between 2010-1990 in Razi Vaccine and Serum Research Institute, were studied.

Materials and Methods

Vaccinal strains of Harvard *C. tetani* (CN49205) were selected. Afterward, vaccinal Harvard strains that were used to prepare the tetanus toxoid in Razi Institute, during the 1990 to 2010, were studied.

Medium culture and conditions of bacterial subculture: All cultures were incubated at 35˚C in an atmosphere

containing 5% H_2, 85% N_2, and 10% CO_2 using an anaerobic cabinet for 72 h. This process complies with safety issues done under laminar hood. Tiny and irregular colonies were grown on Columbia blood agar medium after 72 h. Full loop of bacterial colonies was picked by using a sterile plastic Anas under secure conditions and gently transferred to thioglycolate broth medium. After of 36 h, 1.5 ml of thioglycolate broth medium transferred to a microtube.

Determination of bacterial concentration: A certain concentration of bacteria is required for DNA extraction. By using spectrophotometer at a wavelength of 600 nm, OD (absorption) of bacteria was about 1.1-9.0. Inactivation of bacterial cells was performed for 60 minutes at 80˚C. Then, centrifuge was performed after washing the cells with phosphate-buffered saline (PBS) and about 400 µl of TE buffer was added to microtube.

DNA extraction: DNA extraction was performed by phenol-chloroform method using proteinase K. Quantity and quality of DNA were evaluated for PCR and RFLP tests. This study was conducted to determine the molecular identity of that isolates and the following primers were designed and used. Primer (F): 5'- GCA CAG TAT CAC CGC TAG CTT-3', Primer (R): 5'- GCG TTG GCT GCA TCT TAT TT -3'.

2.5 ml of *C. tetani* lysed cells was added to a PCR containing 4 µl of each primer (5 pmol/µl), 5 µl (10X buffer+ $Mgcl_2$), 1.25 µl dNTP (2.5 mM) and 0.4 µl of DNA polymerase (1 unit/µl).

Cycling conditions used were as follows: an initial denaturation of 95˚C for 10 min. followed by 25 cycles each consisting of 1 min at 94˚C, 1 min at 52˚C, and 1.5 min at 72˚C. Finally, PCR products were electrophoresed and obtained images were analyzed and interpreted.

In this study for molecular identification of vaccinal *C. tetani* strains by RFLP method, 3 µg of samples DNA was cut by PvuII enzyme and were spent in benmari for overnight in 37˚C. This enzyme are identified and cut specific sites. Thos site are CAG CTG and GTC GAC. Electrophoresis of digested DNA was performed to separate DNA fragments in gel agarose. Also DNA size markerII (that labeled with Digoxigenin) was used to determine the bands size. Afterward, by Southern blotting techniques, the gel bands were transferred to a positive charge nylon membrane.

DNA was fixed on the membrane. Pre-hybridization and hybridization was down by digoxigenin labeled probe. Therefore, the membrane was stood in the Pre-hybridization solution for 2-4 hours at 55˚C in the oven. Subsequently, the membrane was soaked in the hybridization solution that have labeled probe, in 65˚C for overnight. Membrane washing was done. Detection was performed using anti-digoxigenin antibody with HRP (Horseradish Peroxidase), NBT (nitro-blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3'-indolyphosphate) Substrates. The data obtained from the isolates were analyzed and compared by Gel pro Analyzer software.

Results

Quality of the extracted DNA, were confirmed with gel agarose electrophoresis. As well as DNA quantitative of these strains was conducted by the Nanodrop device and was found suitable for RFLP.

PCR product was needed to prepare the probe, it is also were prepared with designed primers. By accomplished PCR for those primers**,** 185 bp bands were detected on the gel. Figure 1 successfully, RFLP technique was performed for Harvard *C. tetani* strains that were used to the production of tetanus toxoid to past two decades in Razi Vaccine and Serum Research Institute. In Figure 2, electrophoresis image of Harvard strain digested DNA that was affected by PvuII enzyme, were observed. Figure 2. After Southern blotting technique and transmission the bands from the gel to membrane, hybridization was performed by prepared probes. After appearance of bands on the membrane, just a band was identified by probs. In figure 3 this band can be seen for all tested Harvard strains.

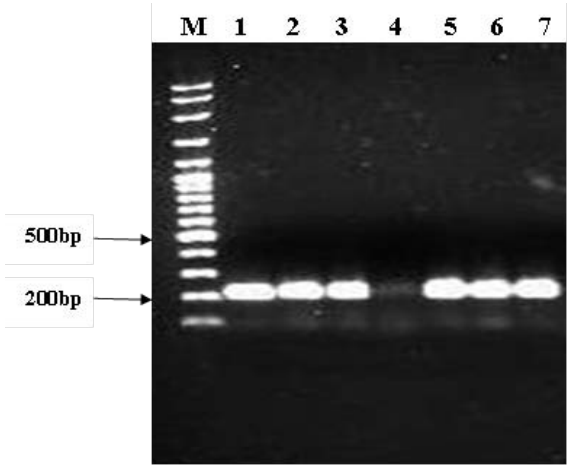

Figure 1. Electrophoresis image of the 185 bp fragment of PCR product in Harvard C. tetani strain for use in RFLP technique. M: marker (100 bp), 4: negative control

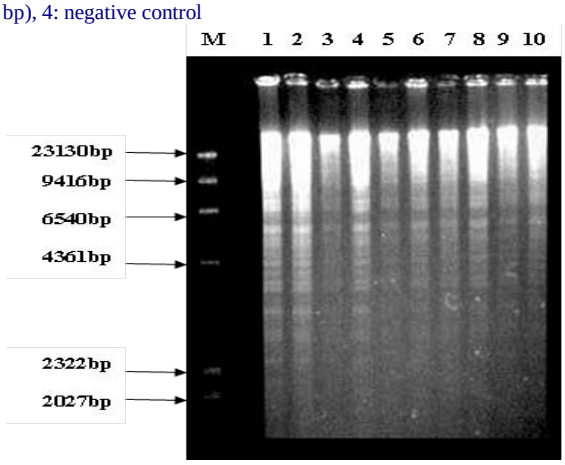

Figure 2. Enzymatic digestion of Harvard C. tetani DNA by Pvu-II, M: marker dig labaled II (roche), 1-10: DNA Enzymatic digestion samples from Harvard Clostridium tetani in different year, 1, 2: (2010), 3: (1990), 4, 8: (2004), 5: (1998), 6, 7: (2001), 9, 10: (2007).

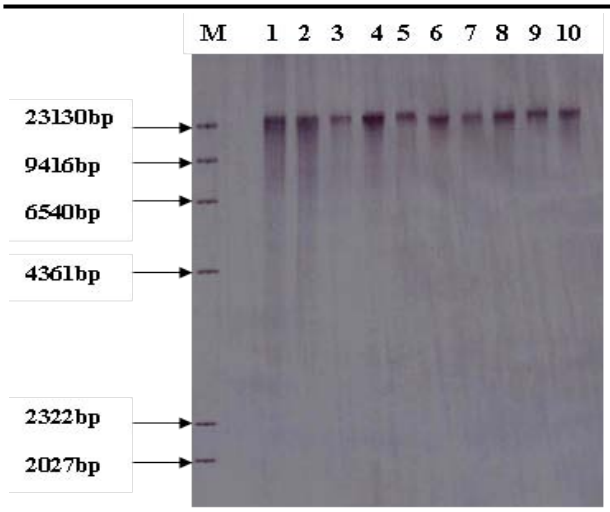

Figure 3. Image of Harvard C. tetani bands on the cellulosic membranes after Southern blotting and RFLP by PvuII enzyme. M: marker dig labaled II (roche), 1-10: Harvard C. tetani bands in different year, 1, 2: (2010), 3: (1990), 4, 8: (2004), 5: (1998), 6, 7: (2001), 9, 10: (2007).

Discussion

In the present study for the first time in Iran, genomic fingerprints were taken for Harvard vaccinal strains of *C. tetani* by applying RFLP technique. Also, in this project, in addition to genomic fingerprinting of mentioned strain, safety of this bacterial strain that used in the preparation of human tetanus toxoid during the 1990-2010, was proved.

Therefore, the tested strains, has not lost its efficiency in production of tetanus vaccine. Tetanus is a frequently fatal disease caused by different *C. tetani* strains. Before tetanus vaccine production, many people were affected by this disease because the bacterial spores are ubiquitous in the environment. There is a permanent risk of contamination with bacteria. Complete immunity by vaccination against tetanus does not exist. Therefore, the need for research and study of these bacteria is very important. The genetic and molecular analysis of the bacteria is one of the best fields of study.

In international conferences and articles are not recorded reports or research in this context yet. Further studies were performed on the *C. botulinum*, *C. difficile*, *C. perfringens* and several other Clostridium species bacteria, but there is not much information about *C. tetani* bacterium. One reason for the lack of interest from scientists and researchers to study this bacterium is the hard work and a lack of sufficient genetic information about it. In the research was conducted in 1991 by Bowman et al., several molecular typing was used for *C. difficile* strains by RFLP method [5].

Gottschalk et al. conducted a survey, in that, the genome sequence of *C. tetani* bacteria were examined [6]. Also, a research was designed by Rokbi et al, in France Pasteur Institute, the relationship between tetX gene and pathogenesis of tested strains was shown [7]. Sarker was studied human *C. perfringens* isolates. Also genomic fingerprinting of these strains was performed by RFLP technique. In this study, Beta 2 gene was used that produce toxin [8]. Also, Katsuyuki et al., was conducted genotypes identification for *C. botulinum* strains that produces type A neurotoxin, by PCR-RFLP analysis [9]. Corry et al. was conducted a study on the uncooked meat in vacuum bags, in that was detected cold bearing temperature Clostridiums such as *C. estertheticum* by T-RFLP method [10]. Usually, in molecular typing methods such as RFLP, cannot be definitively concluded that the mutation or genetic change has occurred in the studied strains. But, were studied the lack of genetic variation in the investigated strain genome by the specific using probes. It is suggested that, studies must be accompanied by the application of other molecular typing such as VNTR (Variable number tandem repeat) and PFGE (Pulsed Field Gel Electrophoresis) for the bacteria to be done. Also, it is better to use other more sensitive probes for RFLP. In addition, any change in the genotype of *C. tetani* genome should investigate. This is being done in Razi Vaccine and Serum Research Institute.

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Authors' Contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

Conflict of Interest

The authors declare no conflict of interest.

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References

1. Rood J, McClane BA, Songer JG. Clostridium tetani and Clostridium Botulinum. In: Titball RW. The clostridia: Molecular biology and pathogenesis. London: Academic Press; 1997: 260-295.
2. Guilfoile P, Babcock H. What Is Tetanus. In: David Heymann. Tetanus. New York: Chelsea House; 2008: 10-45.
3. Laird WJ, Aaronson W, Silver RP, et al. Plasmid associated toxigenicity in Clostridium tetani. J Infect Dis 1980; 142(4): 623.
4. Hara T, Matsuda M, Yoneda M. Isolation and some properties of nontoxigenic derivatives of a strain of Clostridium tetani. Biken j 1977; 20(3-4): 105-15.
5. Bowman RA, O,Neill GL, Riley TV. Non-radioactive restriction fragment length polymorphism (RFLP) typing of Clostridium difficile. FEMS Microbiol Lett 1991; 63(2-3): 269-72.
6. Gottschalk G, Bruggemann H, Baumer S, et al. The genome sequence of Clostridium tetani, The causative

agent of tetanus disease. Proc Natl Acad Sci USA 2003; 100(3): 1316-21.
7. Rokbi B, Baudin MA, Moste C, et al. Molecular characterization of Clostridium tetani strains by pulsed-field gel electrophoresis and colony PCR. Appl Environ Microbiol 2005; 71(9): 5604-6.
8. Sarker MR, Harrison B, Raju D, et al. Molecular characterization of Clostridium perfringens isolates from humans with sporadic diarrhea: Evidence for transcriptional regulation of the beta2-toxin-encoding gene. Appl Environ Microbiol 2005; 71(12): 8362-70.
9. Katsuyuki I, Hiroyuki N, Takayuki K, at al. Genotypic characterization of Clostridium botulinum strains producing type A neurotoxin complexes by random amplified polymorphic DNA and PCR-RFLP analysis. Japanese J Food Microbiol 2005; 4(2): 148-54.
10. Corry JE, Cavill L, Helps CR, at al. Detection of cold-tolerant clostridia other than Clostridium estertheticum in raw vacuum-packed chill-stored meat. Food Microbiol 2011; 28(5): 957-63.

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