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Genetic Structure Change in Harvard Vaccine Strain of Clostridium Tetani for the Period of 1990 to 2010 by Pulsed-Field Gel Electrophoresis

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Abstract

***Background*:** PFGE facilitates the differential migration of large DNA fragments through agarose gel by constantly changing the direction of the electrical field during electrophoresis. Possibility of high difference between strains and repeatability make PFGE one of the strong molecular methods in study of bacterial strains in epidemiology. To identifying and DNA fingerprinting of vaccine strain of *Clostridium tetani* by PFGE technique. Also, possibility of genotyping profile changes in frequency of vaccine strain of *C. tetani* during the period of 1990 to 2011.

***Materials and Methods*:** The vaccine strain of *C. tetani* was provided by Razi Vaccine and Serum Research Institute in Karaj. The seeds were inoculated into Columbia blood agar and grown for 72 h. The cultures were incubated at 35°C in anaerobic conditions. The PFGE analyses were performed using genomic DNA digested with the restriction enzyme SmaI. The electrophoresis analyses were carried out on a CHEF DR III apparatus (Bio-Rad) and band patterns obtained were then analyzed.

***Results*:** The PFGE profile obtained from vaccine strain during a period of more than two decades revealed no remarkable genetic changes and mutations. This type of analysis provides detailed data useful for surveillance of vaccine strains and isolates as well as for the selection of certain predominant profiles for further investigation.

***Conclusion*:** This study showed no considerable change in chromosomal genome of Harvard, the vaccine strain. It is therefore concluded that the vaccine produced by Razi Institute had evidently no alteration or modification in accordance to PFGE profile analysis during a period of more than two decades.

Introduction

Tetanus is a bacterial infectious disease that is caused by *Clostridium tetani*. This illness can be created in all age groups. Despite modern intensive care, nowadays, mortality of this disease is high. This disease remains as a major public health problem in most parts of the world [1].

In many countries, neonatal tetanus is responsible for half of infant mortality that due to vaccine-preventable diseases and approximately 14% of the total infant mortality. Agent of this disease is not the bacteria themselves, but upon the bacteria entering the body that secretes the toxin. With the gradual release of toxins in the human body, painful muscle cramps and severe tetanus disease is the major symptom occurs. Muscle cramps, gradually expands with the progress of the toxin in the body and the muscles involved in breathing and respiratory obstruction, the patient will lead to death [2].

Clostridium tetani is an obligatory anaerobic, Gram-positive, thin and susceptible to heat, movable with terminal or sub terminal spore. These bacteria threaten community groups that are associated with soil contaminated with metals, such as farmers and blacksmiths [3, 4].

Different methods for molecular typing of pathogenic bacteria are used. One of those methods is PFGE (Pulsed-Field Gel Electrophoresis) technique. This method is perfect and confined pattern for demonstrate genomic DNA. This technique because of possible due to the high differentiation between strains, good repeatability and easy exposition is used now widely and as one of the most powerful tools in molecular epidemiology of bacterial strains is accepted. With this manner, possibility of understanding sources of infection and contamination by organisms, genetic diversity and genetic distance between strains is facilitated. Also, PFGE can show the relationship between bacterial isolates clearly. PFGE technique (after sequencing technique) is the second method proposed as a standard typing.

Variety of complex PFGE devices has been reported that the most important factor in making this system is a uniform electric field, for better separation of large DNA fragments have been. In PFGE are used two variables of

the electric field that make an angle with each other. The sum of the fields can be determined direction of movement DNA molecules through the gel. When direction of electric field will change frequently, the movement direction of DNA molecules will changes. The larger DNA molecules needs more time to change direction [5].

The purpose of this study is genomic fingerprinting and study the genomic structure change of vaccinal strain of Harvard *C. tetani* by using PFGE molecular methods. Also, study of mutations and other genetic changes in vaccinal strains used for production of tetanus vaccine in Razi Institute of Karaj, during 1990-2010, is another important aim.

Materials and Methods

Vaccinal strains of Harvard *C. tetani* (CN49205) were selected, initially. Afterward, vaccinal Harvard strains that were used to prepare the tetanus toxoid in Razi Institute during the period of 1990 to 2011, were studied.

All cultures were incubated at 35°C in an atmosphere containing 5% H2, 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 a microtube that containing 400 µl TE buffer (10 mM Tris-HCl, 1mM EDTA pH=8).

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. 150 µl of layzis buffer I (6 mM Tris-Hcl [pH=7.4], 1 M NaCl, 10 mM Na_2EDTA, 0.5% Brij 58, 0.2% Na-deoxycholate, 0.2% Na-lauroylsarcosine) was added to the cell suspension. Adding of 40 u mutanolysin enzyme is essential. Cell suspension was mixed 1:1 with 2% agarose «LMP agarose» and agarose plugs were prepared. The embedded cells were lysed in buffer I supplemented with 5 mg/ml lysozyme and 1 µg/ml RNase for overnight at 37°C. After that, layzis buffer I was replaced by 5 ml layzis buffer II (10 mM Tris-Hcl [pH=7.4], 1 M Na_2EDTA, 1% Na-lauroylsarcosine, 1 mg/ml proteinase K) and the pluges were incubated overnight at 50°C. A piece of the plug (4×4 mm) was cut and transferred to a sterile microtube in sterile conditions (heat and alcohol). Agarose pluges were digested overnight at 30°C with 3 µl of SmaI (To prepare the buffer and enzyme to each sample, 97 µl of enzyme buff and 3 µl of SmaI enzyme was mixed).

Pluges was placed into the gel electrophoresis well. Electrophoresis was performed on 1% PFGE agarose using a CHEF DR III. Running condition were 1 to 20 s. using switch times of 1 to 20 s for 27 h at 6 V/cm in 0.5X TBE (Tris base buffer 54 g, Boric acid 27.5 g, EDTA 4.65 g).

Results

Vaccinal strains of *C. tetani* were selected. After preparation and plaque formation, DNA was digested with SmaI restriction enzyme. After this, electrophoresis was performed in a CHEF apparatus. And, successfully PFGE technique was performed for Harvard strains of *C. tetani* that collection during two decades and was provided by Razi Vaccine and Serum Research Institute in Karaj. Figure 1 shows electrophoresis images of Harvard strains digested DNA that was influenced by SmaI enzyme. As can be seen in the image, each gel well, is related to a specific year. By comparing PFGE profiles that obtained from DNA of human tetanus Harvard vaccinal strains, were observed same molecular pattern for these strains in different years. So tested Harvard strains, showed a similar and identical genetic pattern.

The number of bands observed in the bonding pattern of strains of Harvard *C. tetani* had 13 bands. Also,λ phage, which is a specific marker for PFGE was used in this test. Bands size obtained with this marker. Compared and analyzed by special software, such as Gel pro Analyzer was done.

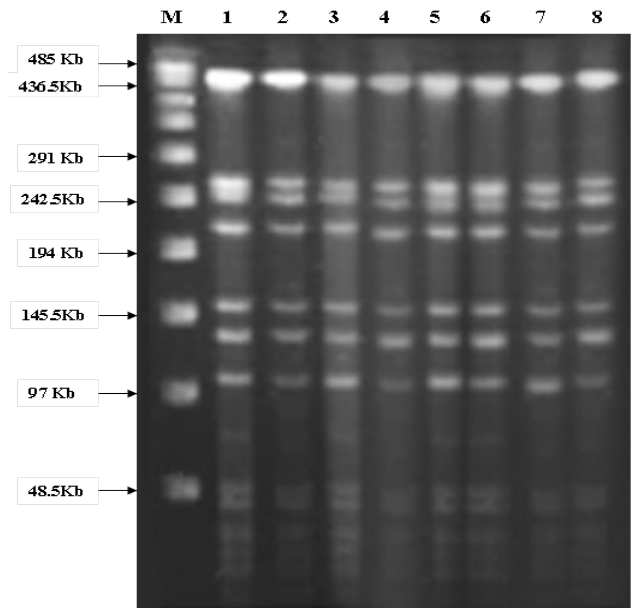

Figure 1. PFGE DNA profiles that obtained from Harvard vaccinal strains of C. tetani by CHEFIII device in different years and have been digested by the SmaI restriction enzyme. M: λ phage marker, lane 1-8: Harvard human tetanus strains in different year. lane 1: 2010, lane 2: 2004, lane 3: 2001, lane 4: 1998, lane 5 & 6: 2007, lane 7 & 8: 1994.

Discussion

In the present study for the first time in Iran, genomic fingerprints were taken for Harvard vaccinal strains of *C. tetani* by applying PFGE technique. According to the results that obtained from this method, displayed no

remarkable genetic changes and mutations in vaccinal strains, between 1990-2010. Furthermore, these studies did not show any heterogeneous profiles. So tetanus vaccines produced in Razi Vaccine and Serum Research Institute at the mentioned period, are safe and healthy. Tetanus disease is not transmitted from one person to another. Also, Environment is reservoir of bacteria that causing to this disease. In developed countries the disease affects mainly adults, because young people have been vaccinated against the disease. Total tetanus deaths, is estimated at about 213 thousand cases in the world, which 180 thousand cases is related to Neonatal tetanus. Mortality from neonatal tetanus is about 15000 to about 30000 cases [6]. In the United States, with an annual incidence of about 100 cases, the disease is rare disease and low number of patients, ultimately die. The best way to deal with tetanus is prevention. For prevention, tetanus vaccination is done. This includes getting vaccinated 3 times, that starts in 2 months and Booster at 18 months, 5 years and then every 10 years will continue [2].

The possibility of permanent infection with tetani bacteria makes the research and studies about this bacterium necessary. One of the most important fields of study is the genetic and molecular analysis of this bacterium.

Johnson et al., was performed Genomic analysis of *C. botulinum* type A by PFGE technique [7]. The studies was conducted by Klaassen et al., regarding epidemiology relationship of nosocomial *C. difficile* isolates by two techniques, PFGE and AFLP (Amplified Fragment Length Polymorphism) and compare its results with each other [8].

Nevas et al. were studied the genetic diversity of strains of proteolytic *C. Botulinum* by PFGE techniques. In this study 55 strains of bacteria with different types A, B, AB, F was used and variety of restriction enzymes was tested [9].

Also, a research was designed by Rokbi et al., in Pasteur Institute in France. Molecular detection technique, PCR and PFGE was performed by the *C. tetani* bacterium. In the PCR experiment, the relationship between tetX gene and pathogenesis of tested strains was shown. Also, several of the *C. tetani* strains and two clinical isolates were analyzed by PFGE technique [10].

Be compared Rokbi methods with used method in this study, were obtained similar results for Harvard *C. tetani* strains. In Rokbi manner, results of several different strains of *C. tetani* and a few clinical isolates have been compared by producing primers and probes of tetX gene. In this research strains quantity was not desired. But only Harvard strains that used in Razi Vaccine and Serum Research Institute in Different years, has been studied. Therefore, extensive research was performed on the genome of this strain to examine safety and health. Also, the genome of this strain was checked for probability mutations.

In this study the CHEF method was used for PFGE testing. This method is modified PFGE type that was invented in 1986 by Chu et al. In this method the point electrodes, placed in a hexagonal chamber surrounding and connected to predetermine potential. The uniform electric field generated in the gel and provides 120 degree angle in the interaction. Also, there is the possibility of separating the samples in a straight direction. One advantage of this method is the change in electric field, which can also be used to isolate molecules smaller than 100 kb [5].

PFGE is one of the most prestigious manners of molecular typing methods. But probability, small mutations are not detected by this method. Therefore, it is suggested that further studies be used in conjunction with other molecular typing methods such as VNTR (Variable number tandem repeat) for the bacteria to be done.

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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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