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Genetic analysis of clinical and vaccine strains of *Bordetella pertussis* by Pulsed-Field Gel Electrophoresis (PFGE), Multi Locus Sequence Typing (MLST) and serotyping

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ABSTRACT

In spite of high vaccination coverage in the Expanded Program of Immunization (EPI), pertussis has not been eradicated yet and the re-emergence of the disease is still reported worldwide. The genetic divergence study of circulating clinical strains of *Bordetella pertussis* among the population with high vaccination coverage is a useful tool to have an insight in the understanding of genetic patterns of this bacterium and deviation of them from vaccine strains. Different methods are accessible for studying of *Bordetella pertussis* that can perform appropriate assessment between populations. Strains used in this study were a collection of two pertussis vaccine strains used to create killed pertussis vaccine over years at Razi Vaccine and Serum Research Institute, 10 clinical and 2 reference strains (ATCC9797 and Tohama I) in Multilocus Sequence Typing (MLST), Pulsed-Field Gel Electrophoresis (PFGE), and serotyping. The genetic profiles of vaccine working and master seeds showed no important change(s) in frequencies of fingerprint types investigated in the vaccine strains and had homogeneity in PFGE method where the clinical isolates showed diversity in genetic profile. Serotyping method showed that all of 10 clinical strains expressing Fim 3. In MLST study, seven housekeeping genes including adk, pgm, fum C, tyr B, gly A, pep A and icd were analyzed which showed no changes in the sequence of clinical and vaccine strains with 100% homology. The genes that cause pathogenicity like ptxC, tcfA and fhaB were also evaluated and the results illustrated heterogeneity in the vaccine and circulating strains.

1. Introduction

The whooping cough disease is still a health problem throughout the world, even though vaccination against it has been used for more than 50 years [1].

Bordetella pertussis is a Gram-negative bacterium and produces many virulence factors that are reasoned the clinical appearance of the disease [2–4]. The virulence factors of *Bordetella pertussis* are divided into two groups, toxins and adhesions. The toxins are such as pertussis toxin (Ptx) [5] and adenylate cyclase toxin (ACT) [6], and attachment factors of pertussis or adhesions are such as filamentous hemagglutinin (FHA) [7,8], fimbriae (Fim) [9], and pertactin (PRN).

The best approach to prevent pertussis is vaccination which has been begun since the 1940s. Primitive vaccines were whole-cell pertussis (WP) formulated as part of the combined diphtheria-tetanus-pertussis (DTP) vaccine. In recent decade, the whole-cell pertussis vaccines have been replaced by acellular pertussis (aP) vaccines in many countries except in many developing countries [10].

DTP vaccination against the pertussis was started in Iran since 1950 using vaccines manufactured by Razi Institute (DTwP) after approval and conducted by the National Committee on Vaccination. The capability and efficacy of the vaccine by previous studies showed the detoxified and inactivated pertussis vaccine introduced, had successfully handled the occurrence of pertussis ever since. Reports by WHO (World Health Organization) have shown that diphtheria and tetanus toxoids and pertussis (DTP) coverage is greater than 96% in Iran since 2000 [11].

In recent years, a resurgence of pertussis particularly among adolescents has been seen in many industrialized countries with a high immunization coverage [12]. The located shift in pertussis epidemiology can be an outcome of pertussis reportage, host elements, waning vaccine-induced immunity, demographic changes, vaccine coverage changes, vaccine effectiveness changes and adaptation of the *Bordetella pertussis* population to vaccine-induced immunity [13].

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DNA distance-based phylogenetic methods are available for characterization of B. pertussis isolates such as Multilocus Sequence Typing (MLST) [14], IS1002-based fingerprinting [15,16] Whole-genome DNA Microarray [17,18], Variable-Number Tandem Repeat (MLVA) [19,20] and Pulsed-Field Gel Electrophoresis (PFGE). At present, the most proper way of DNA fingerprinting of *Bordetella pertussis* is PFGE which is the most reliable and suitable method, as it has been appeared to have a more discriminatory power in comparison to the other techniques, even if it is difficult to perform [13].

Serotyping is one of the earliest techniques used for *Bordetella pertussis* typing and was advancing at a time when the number of methods available for characteristic bacterial isolates had limited [13,14]. The stereotype seems to change with population immunity, and serotyping has provided data to suggest that defenses towards whooping cough depend on the stereotype particulars of the pertussis bacteria [21]. This is obvious when vaccines lacking one serotype given to a population, lead in pertussis cases brought on by the strains of the serotype not within the vaccine preparation [22–24].

Originally, many various stereotypes were used to diagnose *Bordetella pertussis* isolates and characterization was made only between serotypes 1, 2 and 3. The antigen of serotype 1 is part of the *Bordetella pertussis* Lipo-oligosaccharide and is not appropriate for discriminating between *Bordetella pertussis* isolates as it does not vary. In this study, serotyping was done by monoclonal slide agglutination assays.

The diversity of B. pertussis strains has been studied by genes sequencing encoding superficial proteins. Of these, genes including ptxA, ptxC, fhaB, prn, fim2, fim3 and tcfA had single nucleotide polymorphism. Multilocus sequence typing (MLST) has been released as a new way for studying of the molecular epidemiology of bacterial pathogens [25,26]. MLST is established on the well-tested principles of multilocus enzyme electrophoresis, and assigns alleles at each site directly by nucleotide sequencing instead of indirectly from electrophoretic mobilities with their gene products in starch gels [27].

Due to the high incidence and different circulating strains in the world, it is necessary to specifically analyze genetic patterns of these isolates associated with sequence analyses of effective factors in protection. In this study, PFGE, MLST and serotyping were employed to study the circulating strains in Iran, vaccine working and master seeds in Razi Vaccine and Serum Research Institute.

2. Material and methods

2.1. Bacterial strains and culture

In the current study, 10 clinical samples collected from different regions of Iran such as Tehran, Qom, Khuzestan, Esfahan, East and West Azarbaijan (Table 1), six vaccine strains included 134 and 509 working and master seeds used in the preparation of the (DTP) vaccine between 2000 and 2014 at Razi Vaccine and Serum Reseach Institute and two reference strains (Tohama I and ATCC 9797) were used for validation. All strains were studied and approved by methods of Gram-staining, biochemical tests and by IS1001, IS1002 and IS481 using Real-Time PCR (primers and probes are available in Table 2). Bacterial seeds were cultured onto Bordet-Gengou Agar (BGA) supplemented with 20% defibrinated sheep blood and 1% glycerol in a humidified condition incubated at 35 °C for 3–5 days.

Table 2
Primers and probes used in Real-Time PCR for amplification of the insertion sequences IS481, IS1001 and IS1002.

Pertussis IS481	F- CAAGGCCGAACGCTTCAT
	R- AGTTCTGGTAGGTGTGAGCGTAAG
	Probe 1: BPpr-FLU
	5'–CAT GCG TGC AGA TTC GTC GTA CA(F)
	Probe 2: BPpr-LCR
	5'–(LC-Red 640)ACC CTC GAT TCT TCC GTA CAT CC(P)
Pertussis IS1001	F- CCGGCTCGACGAATTGC
	R- AGAACCAGAGCCGTTTGAGTT
	Probe 1: BPpara-FLU
	5'– GTT CTA CCA AAG ACC TGC CTG GGC-(F)
	Probe 2: BPpara-LCR
	5'– (LC-Red 640) AGA CAA GCC TGG AAC CAC TGG TAC-(P)
Pertussis IS1002	F-CTAGGTCGAGCCCTTCTTGTTAAC
	R-GCGGGCAAGCCACTTGTA
	Prob:Hex-CATCGTCCAGTTCTGTTGCATCACCC-BHQ1

DNAs were extracted from bacterial suspensions then Real-Time PCR was performed using primers and probes shown in Table 2. Grade water and B. pertussis DNA were used for the negative and positive control, respectively. Amplification curves were drawn up and results analyzed (Guidance and protocol for the use of real-time PCR in laboratory diagnosis of human infection with *Bordetella pertussis* or Bordetella parapertussis)

2.2. PFGE

2.2.1. Preparation of DNA plugs

After culturing bacteria on BGA medium, bacterial suspension was prepared by TE1X (10 mM Tris − HCl at pH 8, 1 mM EDTA) to obtain a bacterial suspension containing 10^9 cfu/ml (OD = 0.66 at 650 nm). One-percent low melting-point was then mixed in TBE 0.5X in a ratio of 1:1. Subsequently, 300 ml of the mixture was poured into plug mold and then cooled until solidified.

The plugs were placed in 1 ml lysis buffer containing 2.5 λ proteinase K per ml, 0.5 M EDTA at pH 8 and sarkosyl 1%. The plugs were incubated overnight in a water bath at 55 °C and afterward incubated for 1 h at 55–57 °C with 30 ml TE 1X and 300 λ of a phenyl-methyl-sulfonyl-Fluoride (PMSF). The tubes were then cooled on ice and washed with 10 ml TE 1X buffer at pH 8.

2.2.2. Restriction enzyme analysis of DNA plugs

The plugs were cut into two pieces. Slices were incubated 3 h at 37 °C in 100 ml 1X buffer solution (manufacturer's instructions) containing 30 U of XbaI. As fragments of less than 30 kbp can diffuse out of

Table 1
Location of isolations, age, sex, history of vaccination, history of antibiotic used, symptoms and culture.

Number	Location	Age	Sex	Sample	Vaccination	Taking antibiotics	Symptoms	Culture
1	Tehran	2.5 months	F	nasopharynx	+	+	1,2	positive
2	North West of Iran	2.5 months	M	nasopharynx	+	+	1,2,3	positive
3	Tehran	2.5 months	F	nasopharynx	+	+	1	positive
4	Esfahan	10 years old	M	nasopharynx	+	–	1,2,3	positive
5	Khuzestan	40 days	M	nasopharynx	–	+	1,3	positive
6	Qom	3 months	F	nasopharynx	+	+	1	positive
7	East Azarbaijan	6 months	M	nasopharynx	+	+	1,2	positive
8	East Azarbaijan	1.5 months	M	nasopharynx	–	+	1,2	positive
9	Zanjan	1.5 months	F	nasopharynx	–	–	1,2,3	positive
10	North west of Iran	4 months	F	nasopharynx	+	Unknown	1	positive

Symptoms of the disease in isolates. 1 = indicates the presence of cough, 2 = indicates the presence of nausea, 3 = indicates the presence of fever.

the plug, storage of very small fragments was not recommended.

2.2.3. Electrophoresis

To prepare agarose gel, 1gr melted agarose were solved in 100 ml TBE 0.5 × . The CHEF DR III apparatus with a cooling module (Bio-Rad, USA) were used. The electrophoresis unit was filled with 2.2 liters of TBE 0.5X, and the cooling system set at 14 °C. The program was set according to a migration period of about 24 h as follows: ramping 5–6 s, 5.5 V/cm, 5 h and ramping 8–35 s 5.5 V/cm, 19 h. In each run, two reference strains and Lambda markers were loaded on the gel.

2.2.4. Ethidium bromide staining

After electrophoresis, the gel was stained by 1 mg/ml ethidium bromide and put in a water bath for 20 min and rinsed gently with tap water.

2.2.5. Technical validation

All runs were confirmed by comparing the band patterns of two reference strains (ATCC9797 and Tohama I) on the gel.

3. Serotyping

Serotyping was performed by a microtiter plate-based and achieved using monoclonal antibodies to agglutinogens 2 and 3, such as Anti-Agglutinogen 3 *Bordetella pertussis* 3 (WHO reference reagent, anti-B. pertussis fimbriae 3 monoclonal antibody, NIBSC code: 04/156) and Anti-Agglutinogen 2 *Bordetella pertussis* (WHO reference reagent, anti-B. pertussis fimbriae 2 monoclonal antibody, NIBSC code: 04/154, UK), respectively. The validity of the results was proved by using negative and positive controls. The positive controls were considered as *Bordetella pertussis* vaccine strains 509 and 134, expressing Fim2 and Fim3, respectively. The deionized water was used as negative control.

4. MLST

4.1. DNA extraction

18 samples were tested with different age, sex and vaccination history regarding geographical distribution. DNA extraction was performed using High Pure PCR Template Preparation kit (Roche Company - Germany) from microbial suspensions of single colonies to the OD of 0.6 in 600 wave lenght.

4.2. PCR

Following the culture, DNA extraction and purification; PCR was performed with specific primers (Table 3) for the genes encoding ptxC, tcfA and fhaB as well as housekeeping genes including pgm, adk, fum C, icd, tyr B, pep A and gly. PCR mixtures in a total volume of 25 µl containing 1 µl of each forward and reverse primers, 2 µl of template DNA and 12.5 µl of red master mix (Ampliqon Company- Denmark) were prepared. The cycling conditions for housekeeping genes were as follows: 95 °C for 15 min and 30 cycles of 95 °C for 30 s, 54 °C for 30 s, 72 °C for 1 min [28]. After running the products in 1% agarose gel, associated with DNA Ladder 100 bp plus, each band was investigated.

4.3. Sequencing and data analysing

DNA sequencing was performed using an ABI system (Macrogen Research -Korea) and the sequences were read and edited by Chromas and analyzed using standard MEGA software in conjunction with reference strains, ATCC9797 and Tohama I.

5. Results

To distiguish Bordetella species in isolates, the results of the Real-time PCR for insertion sequences IS481, IS1001 and IS1002 showed the presence of IS481 and IS1002 and no IS1001 that confirmed all the 10 clinical isolates were B. pertussis.

Table 3
PCR forward and reverse primers used in MLST method.

Sequence 5´→ 3´	Gene Name
F→ CTTCCGGAGGTTTCGACGTTTC	ptxC
R→ TCTTTCAAGGGATTCATTCGC	
F→ CTTTCTCCTCCCTCGGCATGG	tcfA
R→ AGCGCCGTCCGGATTCAAG	
F→ GGTTCAGAGCGTCAACAGC	fhaB
R→ CTCACCAGCTTCGCAACG	
F→ CGC CCA TGT CAC CAG CAC CGA	pgm
R→ CGC CGT CTA TCG TAA CCA G	
F→ CGT GAA CCG GGG CCA GTC GTC	fum C
R→ GGC CAG CCA GCG CAC ATC GTT	
F→ AGC CGC CTT TCT CAC CCA ACA CT	adk
R→ TGG GCC CAG GAC GAG TAG T	
F→ CAA CCA GGG CGT GTA CAT GGC	glyA
R→ CCG CGA TGA CGT GCA TCA G	
F→ CGA GAC CTA CGC TTA TTA CGA T	tyrB
R→ TGC CGG CCA GTT CAT TTT	
F→ CTG GTC CAC AAG GGC AAC AT	icd
R→ ACA CCT GGG TGG CGC CTT C	
F→ CGC CCC AGG TTG AAG AAA ATC GTC	pepA
R→ ATC AGG CCC ACC ACA TCC AG	

5.1. PFGE

5.1.1. Clinical and vaccine strains analysis

Genetic fingerprinting results of clinical and vaccine strains were compared in accordance with the genetic pattern (Fig. 1). Strains 1, 2 and 3 (rows 1vac, 2vac and 3vac) having Dice coefficients equal to 100%. Also, in rows 4, 5 and 6 the Dice coefficient was equal to 100% which showed unchanged genetic pattern of the vaccine strains 134 and 509 during the continuous passages for many years. Although two vaccine strains have Dice coefficient up to 65%, suggesting that the two group strains are Bordetella pertussis, but have differences with each other, as it shows in the Fig. 1, the numbers of band observed in the first group of *Bordetella pertussis* to compare with the second group were 20 and 17, respectively.

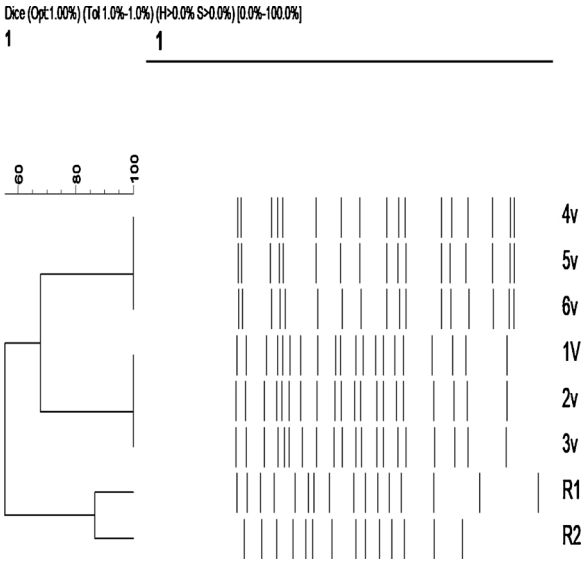

Fig. 1. Dandrogram analysis of six vaccine (vac1-vac6) strains used to produce killed pertussis vaccine during the period of 2000–2014 at Razi Vaccine and Serum Research Institute, Karaj-Iran, and two reference strains (REF1:TohamaI and REF2: ATCC9797).

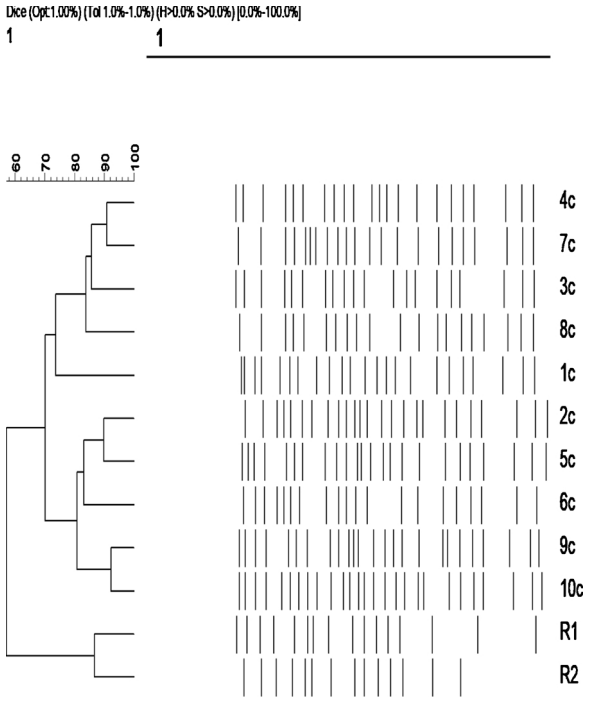

Fig. 2. Dandrogram of 10 pulsed –field gel electrophoresis profile of the Burdetella pertussis circulating isolates in Iran and 2 reference strains (REF1: Tohama I and REF2: ATCC9797).

Dandrogram analysis of six vaccine and two reference strains (REF1:TohamaI and REF2: ATCC9797)

5.1.2. Clinical strains

Clinical isolates numbered 2, 6, 8 and 9 with numbered 1 and 5 had approximately 87% similarity. Clinical isolate numbered 1, 2, 3, 5, 6, 8 and 9 had 80% similarity with strains numbered 7. Moreover, clinical strains numbered 2 and 9 had Dice coefficient of approximately 94% with the isolated clinical strains numbered 6 and 8 which were very closely related to each other, and strains 3 and 4 together had a similarity coefficient of approximately 83%, and its relation to the both previous species was approximately 75% (Fig. 2). The clinical and vaccine strains had hetrogenicity but the percentage of genetic relationship was relatively high 75%.

Dandrogram of clinical and 2 reference strains (REF1: Tohama I and REF2: ATCC9797)

5.1.3. Comparative bonding pattern study between vaccine and clinical strains

Vaccine and clinical strains produced distinct bonding patterns and all had typeability pattern. To draw bonding pattern graphs of clinical and vaccine strains, reference strains ATCC9797 and Tohama I were documented by the software (Gel compare II) and bonding patterns drawn. The bonding patterns of reference strains together had 80% similarity coefficient and clinical and vaccine strains had approximately 56% similarity coefficient together that shows the possible association of these strains (Fig. 3). Vaccine and clinical strains produced distinct bonding patterns and all had typeability pattern.

Dandrogram of comparative bonding patterns of the clinical, vaccine and reference strains (REF1: Tohama I and REF2: ATCC9797)

5.2. Serotyping

It was shown, in this study, that all of 10 clinical strains expressing Fim 3; 3 vaccine strains 509 expressing Fim 2; and 3 vaccine strains 134 expressing Fim 3.

5.3. MLAT

5.3.1. ptxC gene

Results of sequencing for ptxC gene in polymorphic region showed that most of the circulating strains had ptxC2 allele and of minority isolates had ptxC1. Therefore, the dominant allele was the ptxC2 in the recent bacterial circulation. Vaccine B. pertussis strains 134 and 509, and the reference strain ATCC 9797 contained ptxC1. It can be concluded that the dominant allele for ptxC gene is a dissimilar allele in the vaccine strains to compare with isolates in Iranian B. pertussis population (Fig. 4).

PCR of *ptxC* gene

5.3.2. tcfA gene

Results of sequencing for tcfA gene in polymorphic region for clinical isolates showed that all of the circulating strains had tcfA5 allele. Vaccine B. pertussis strains 134 and 509 also showed the same allele as in the isolates (Fig. 5).

PCR of *tcfA* gene

5.3.3. fhaB gene

The fhaB gene has two known alleles, like the ptxC gene. After alignment of the sequences from clinical specimens, standard and vaccine strains and comparing them with alleles introduced for fhaB gene, it was found that the fhaB1 allele had a prevalence with high frequency. The alleles present in the vaccine strain 509 and ATCC9797 strain were fhaB2 and vary with the dominant form of the allele in the bacterial population. The vaccine strain 134 was also similar to most of the clinical specimens containing the fhaB1 allele (Fig. 6).

PCR of *fhaB* gene

5.4. MLST results for housekeeping genes

After performed PCR on housekeeping genes, the pruducts with DNA ladder (DNA Ladder 100 bp plus) were loaded in 1% gel agarose. According to the primers used, the length of the housekeeping fragments was confirmed about 500 base pairs. In the next step, sequences obtained from the present study and their similar sequences were compared to the gene bank. The results obtained in the previous steps are presented as a phylogeny tree. The following results are two examples of the seven housekeeping genes.

5.4.1. tyrB

After sequencing (Macrogene company) and converting sequences to FASTA format, the sequences were analyzed using standard softwares including DNASIS Max, MEGA and Artemis. The sequences obtained from the resulting research and similar sequences in the gene bank were compared, aligned and showed two main branches in phylogenetic tree pattern of the tyrB gene. Also, the standard strains Tohama I and ATCC9797 (18323) are shown in the subcategory (Fig. 7).

5.4.2. glyA

The sequences obtained from the resulting research and similar sequences in the gene bank were compared, aligned and showed two main branches in phylogenetic tree pattern of the glyA gene. Also, the standard strains Tohama I and ATCC9797 (18323) are shown in the subcategory (Fig. 8).

6. Discussion

Currently, whooping cough is one of the ten common causes of deaths from infectious diseases in the worldwide. Regardless of vaccine availability for whooping cough, it still remains a native disease and

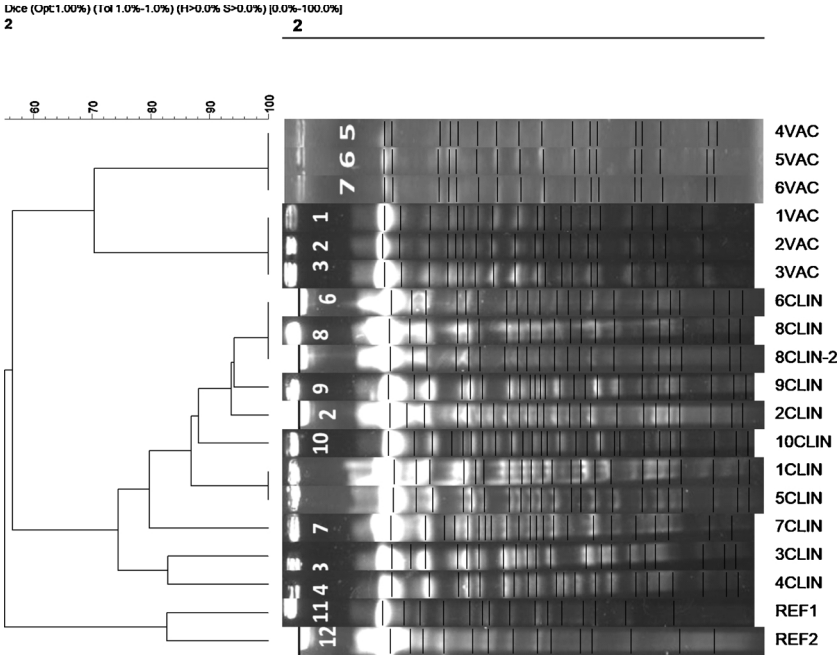

Fig. 3. Dandrogram of 10 pulsed –field gel electrophoresis profile of Burdetella pertussis circulating isolates in Iran, 6 vaccine strains (vac1-vac6) used to produce killed pertussis vaccine during the period of 2000–2014 at Razi Vaccine and Serum Research Institute and 2 reference strains (REF1: Tohama I and REF2: ATCC9797) using gel compare II program.

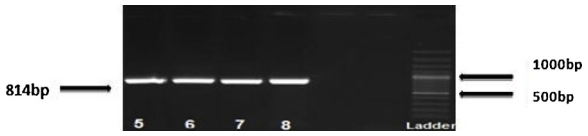

Fig. 4. Electrophoresis of PCR product for ptxC gene in clinical strains 5,6,7 and 8. Ladder size is100bp.

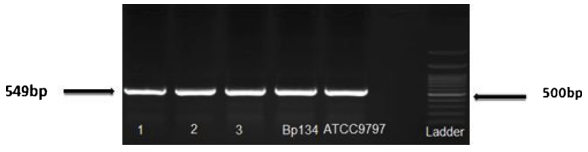

Fig. 5. Electrophoresis of PCR product for tcfA gene in Bp134, ATCC9797 and clinical strains 1,2 and 3. Ladder size is100bp.

Fig. 6. 3 Electrophoresis of PCR product for fhaB gene in vaccine strain 509 and clinical strains 1,2 and 3. Ladder size is100bp.

classified by the CDC as a re-emerging disease [29]. Although the mortality and morbidity rates have significantly reduced by vaccine injection, epidemics occur every 3 to 5 years [13]. In addition, in countries with high vaccination rates, including Australia, Canada, France, Norway, The Netherlands and the United States, there is evidence of increasing pertussis incidence. There are several explanations about the recurrence of pertussis in the vaccinated population. Improvements in monitoring, changes in description and diagnostic methods may appear to increase the incidence of pertussis. At present, the role of vaccine antigen variation in B. pertussis evolution or adaptation to vaccine-mediated immunity is not clear, although new or unusual genetic patterns or changes in the clinical strains were identified by comparing strains from different countries with the history of similar vaccination. After many studies, it was concluded that clear change(s) in the *Bordetella pertussis* population and consequently evolution have been made. Similar studies have been conducted to evaluate the genetic map of *Bordetella pertussis* by PFGE, in the worldwide. Advani et al. [30] studied on clinical isolates of *Bordetella pertussis* between years 1998 to 2009 which the outcome was to identify the common PFGE profiles of B. Pertussis populations circulating in European countries with various vaccine coverage and different vaccination programs [30]. 199 representatives from laboratories involved in epidemiological research on pertussis met at the Institute Pasteur, Paris,

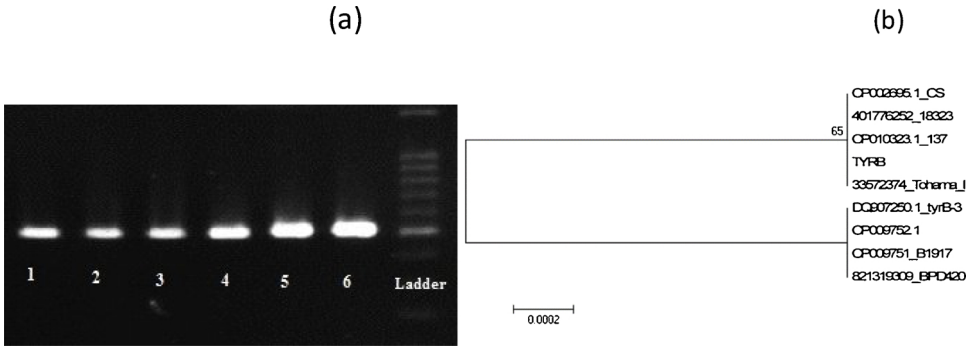

Fig. 7. (a) Electrophoresis of PCR product for tyrB gene; vaccine strains 1 (509), 2 (509), 3 (134), 4 (134), 5 (ATCC9797), 6 (Tohama I); Ladder size is 100bp plus and the length of the housekeeping fragment is about 500 bp. (b) Phylogeny tree of tyrB gene.

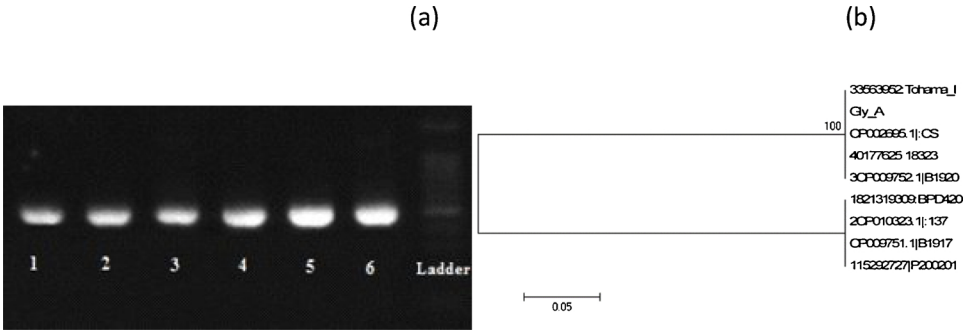

Fig. 8. (a) Electrophoresis of PCR product for glyA gene; vaccine strains 1 (509), 2 (509), 3 (134), 4 (134), 5 (ATCC9797), 6 (Tohama I); Ladder size is 100bp plus and the length of the housekeeping fragment is about 500 bp. (b) Phylogeny tree of glyA gene.

France. The purpose of the meeting was to reach an agreement on the methodology to be applied in the epidemiological study of *Bordetella pertussis* and Mooi et al. [13] introduced PFGE as a standard method to study *Bordetella pertussis* [13].

In Iran, vaccination against DTP has been carried out since 1950s using the vaccine produced by Razi Vaccine and Serum Research Institute (RVSRI), approved by the Iranian National Vaccination Committee, and has been validated by previous studies. Since continuous passages for *Bordetella pertussis* standard strains in vaccine production process is mandatory, the probability of the antigens being lost in the product is high. In the current study, genetic consistency of the vaccine seed strains used in the years between 2000–2014 by RVSRI was studied and the quality of B. pertussis vaccine working seeds showed no sigificant changes in each strain used to produce killed pertussis vaccine.

Strains 1vac, 2vac and 3vac had Dice coefficients equal to 100% and also 4vac, 5vac and 6vac had the Dice coefficients equal to 100%. The number of bands in the first group of *Bordetella pertussis* was 20 bands and banding patterns observed for the second group was 17 with the same genetic pattern. They had Dice coefficient of 65% which suggested these two strain groups are *Bordetella pertussis* nevertheless, they have differences with each other in terms of aiming to produce specific antigens in vaccine production. Circulating isolates had hetrogenisty which was different banding patterns from 1 to 6 together caused probably due to mutations by means of various factors.

In PFGE method, typeability, reproducibility and discriminatory power are important. To consider reproducibility, the clinical strain numbered 8 was tested three times to demonstrate this ability and result of the test repetition showed the same profile (Fig. 9). As it was also shown in Fig. 9, all of the strains were typeable.

Dandrogram of bonding patterns of reproducibility, the clinical strain numbered 8

B. pertussis produces numbers of adhesins at the surface of the organism including fimbriae, filamentous hemagglutinin, pertactin, the serum-resistance protein BrkA and Tcf. Fimbriae of two serotypes, serotype 2 and serotype 3, are produced by B. pertussis and their genes expression are regulated by mutations in their promoter regions which ended in serotype switching. A C-rich region endures relatively frequent deletions and insertions which strongly affects the fimbrial genes transcription. As a consequence, some strains produce only one (serotype 2 or 3), some both types of fimbria (serotype 2 and 3) and some none of them. Presence of agglutinating antibodies are correlated with protection which has led to the addition of Fim2 and Fim3 proteins to the new acellular pertussis vaccines. In no vaccinated populations, it has been shown that the most prevalent serotype was found to be Fim2, whereas in vaccinated pepole the bacteria preferentially express Fim3 [13] The result of serotyping was showed that 3 vaccine strains 509 expressing Fim 2, and 3 vaccine strains 134 expressing Fim 3 and all of 10 clinical strains expressing Fim 3. Over time, the serotypes of *Bordetella pertussis* have therefore changed.

Filamentous hemagglutinin (FHA) is a cell surface protein of *Bordetella pertussis* which antibody produced against this hemagglutinin prevents the bacteria from attachment to the host cell. This protein is an important and highly immunogenic factor in B. pertussis and is considered as the dominant factor for binding. The fhaB gene has two known alleles, fhaB1 and fhaB2. The allele present in the vaccine strain

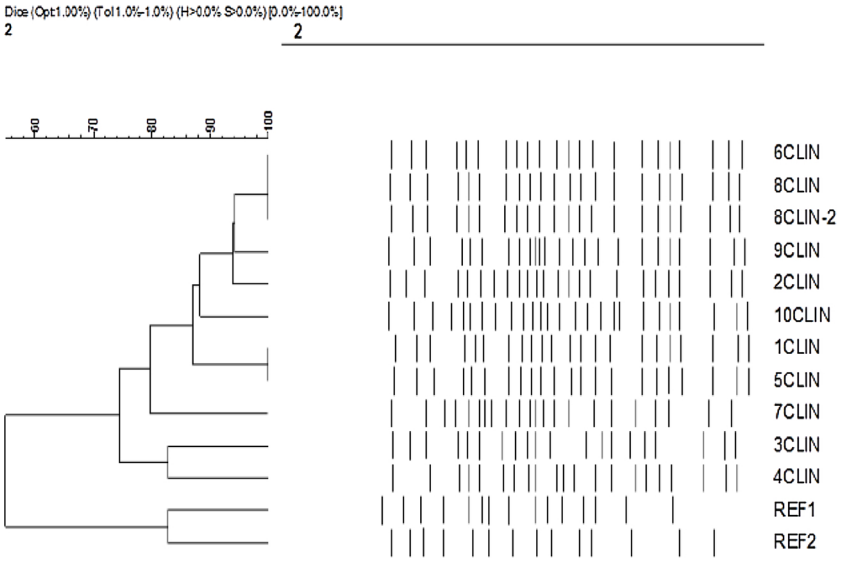

Fig. 9. Clinical strain 8 was tested three times to demonstrate reproducibility characteristic of the strains.

509 and reference strain ATCC9797 was fhaB2 and for vaccine strain 134 was similar to most of the clinical specimens with high frequency containing the fhaB1 allele. Very limited studies have been conducted on the fhaB gene which probably the reason is the gene length and the presence of repetitions that affect the accuracy of sequencing.

Tracheal colonization factor (TcF) is expressed exclusively in B. pertussis and not in other species of Bordetella. It has been shown that this gene has six different alleles. Comparison of the results for tcfA sequencing showed that the dominant allele in isolate strains were tcfA5. The tcfA-5 allele has an extra guanine, leading to a predominant change and a premature end to the translation. The allele in the vaccine strains 509 and 134 was also similar to that of the clinical strains, tcfA5. The reason for the lack of difference in tcfA between vaccine strains and isolates is not known. An explanation in this regard could be that the TcF as an adhesion factor does not endanger the immune pressures that other adehesins may affect.

Pertussis toxin is a 105-kDa protein that produces lymphocytosis and histamine susceptibility and has an A/B structure with a mechanism similar to that of cholera. Two alleles of ptxC is ptxC1 and ptxC2 which differ at a single nucleotide resulting in a silent mutation. In the current study, genetic relationship between different clinical and vaccine strains based on the target region sequences of each of the genes with Neibour joining cluster analysis method was evaluated and phylogenetic tree was drawn up. The dominant allele for circulating strains was found to be 87.5% ptxC2. However, the sequencing results of the targeting gene in the vaccine strains 134 and 509 as well as ATCC9797 indicated that the ptxC1 allele was the only dominant allele. This could be a sign of bacterial compatibility for survival in the human population.

Neighbour joining phylogenetic relationships in the genetic analysis of the ptxC, tcfA, and fhaB sequences, as well as comparing the alleles in these three genes in clinical isolates and vaccine strains, showed that the vaccine strains 134 and 509 had more or less genetic similarity to the circulating strains and may still be used as suitable strains for vaccine production in this area. However, the results for the challenge strain ATCC9797 indicated that this strain was more genetically distinct from circulating and vaccine strains.

Housekeeping genes review showed that the pathogenic strains had no significant changes. However, the polymorphic status of isolates was relatively low, construction of an efficient and continuous surveillance system to detect emergence of genotype variants and confirm interrelationship of genotype change with vaccine immunity is required. Employing dominant B. pertussis isolates into vaccine production, or even using modified versions of current vaccine strains, is a main likely stage to undertake. Nevertheless, it is necessary to understand the effect of antigen variation on immunity in order to notify this process.

The genome of B202 (strain 134) was sequenced and showed phylogenetically and structurally similar, but not identical, to other strains with the profile of prn1-ptxP1-ptxA2-ptxB2-fimH1 [31,32]. Also, whole-genome shotgun sequencing was performed for four *Bordetella pertussis* vaccine reference strains from Serum Institute of India and showed a genome size of 4,128,984 (bp) with prn1-ptxP1-ptxA2 and 4,140,370 (bp) with prn7-ptxP2-ptxA4 for 134 (Accession no: CP017402) and 509 (Accession no: CP017403) vaccine strains, respectively [31,32].

7. Conclusions

We consider that this study could enclose a good basis for further monitoring of the circulating isolates and vaccine strains in Iran to evaluate the impact of the vaccine on genetic changes of circulating strains. It is required to compare more isolates from different parts of Iran to find the exact or whole genetic patterns of the strains. Using of the most circulated genetic patterns of strains for making vaccine in the future, a combination of other techniques in genetic fingerprinting to achieve more acceptable and reliable data for making decision on vaccination program and prohibiting the vaccine import from unknown or unreliable origins with unknown genetic fingerprinting of strains are necessary to avoid an imbalance of circulating with vaccine strains and creating epidemics from current endemic status.

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Ethics

I hereby declare that all ethical standards have been respected in preparation of the submitted article.

Conflict of interest

The authors declare that they have no conflict of interest.

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