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Polymorphism of Virulence Factor Genes *Prn* and *Fim* of *Bordetella Pertussis* Clinical Isolates in Iran

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ABSTRACT

Objectives: *Bordetella pertussis* is a Gram-negative coccobacillus bacterium which is the causative agent of whooping cough. In recent years, the number of whooping cough cases has been rised. This bacterium has important virulence factors such as fimbriae and pertactin. In this study, polymorphism of Serotype 2 and 3 fimbriae genes and 2 Regions of pertactin gene were surveyed.

Materials & Methods: Totally, 20 *B. pertussis* clinical isolates were tested. DNA was extracted using the kit. Serotypes 2 and 3 fimbriae genes and pertactin Region 1 and 2 were identified using PCR method; finally, 13 samples were randomly sequenced.

Findings: No mutation was observed in the pertactin Region 2. In relation to the region1 of pertactin, %77 and %23 of the strains had *prn*2 and *prn*1 alleles, respectively. In relation to fim2 gene, %70 and %30 of the strains had *fim*2-2 and *fim*1-2 alleles, respectively. Also, in relation to *fim*3 gene, %70 and %30 of the strains carried *fim*3B and *fim*3A alleles, respectively.

Conclusion: In general, the present study results were similar to those of the previous studies conducted in Iran, but there were some differences in *fim*2 gene polymorphism so that the dominant allele changed from *fim1-2* to *fim2-2*. Considering the fact that vaccine strains of *Bp134* and *Bp509* carry *fim*3A allele, which is different from the dominant circulating allele (*fim*3B), it is suggested that strains more similar to the dominant circulating strains should be used in designing vaccines.

Keywords:*Bordetella pertussis*; whooping cough; pertactin; fimbriae

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Introduction

Bordetella pertussis organisms are Gram-negative coccobacilli bacteria which are the causative agents of whooping cough [1,2]. Whooping cough or pertussis is a respiratory disease with high severity and acuteness. It has been estimated that about 16 million whooping cough cases and 195,000 deaths occur per year [3,4].

B. pertussis contains some adhesins and virulence factors which enable it to infect, colonize, and attack host cells, including pertussis toxin, adenylate cyclase toxin, tracheal cytotoxin, dermonecrotic toxin, pertactin, and tracheal colonization factor, *bordetella* resistant to killing (BrkA) protein, fimbriae, and filamentous hemagglutinin. Pertactin is encoded by *prn* gene containing an arginine-glycine-aspartate (RGD) motif. This motif is required for *Bordetella* strains to adhere integrin binding sites of host cells. Also, *prn* gene is a component of acellular pertussis vaccines, which has 2 key regions in its structure (*prn*-1 and *prn*-2). Fimbriae (Fim) are proteins mediating adhesion to cell surface, which are necessary for the colonization of *B. pertussis* to respiratory tract cells. *B. pertussis* expresses two serologically distinct fimbriae, including Fim2 and Fim3, which are encoded by *fim2* and *fim3* genes, respectively [5,6].

Whooping cough has 2 main stages including catarrhal and paroxysmal stages. Catarrhal stage is characterized by the disease similar to the common cold. While the cough gradually becomes more severe, paroxysmal stage is begun after 1–2 weeks. In paroxysmal stage, the patient has numerous and rapid coughs and vomiting, and exhaustion happens as usual. This stage usually remains for 2-3 weeks. Pertussis is more severe in infants and children [7].

After the global use of DTP vaccines in 1948, the incidence of pertussis reduced significantly. But after this success, the problem was vaccine side effects, including fever, body redness and swelling, sudden infant death syndrome (SIDS), and so on [2]. In recent years, some countries including the United States, United Kingdom, Australia, and the Netherlands have witnessed an increase in the incidence of whooping cough; in other words, some countries have experienced pertussis outbreak [2], even countries with high immunization rates [8]. The reasons of pertussis resurgence are multifactorial, which are different from one country to another one. For example, some important reasons include improved diagnostic methods, shorter duration of vaccine-induced protection, and relative decline in the efficacy of acellular vaccine compared with the whole cell vaccine, and the likely emergence of vaccine escape *B. pertussis* strains [8].

In a research in the Netherlands, it was suggested that antigenic divergence between the *B. pertussis* clinical and vaccine strains may contribute to the pertussis resurgence. Genetic variations in circulating strains of *B. pertussis* have been observed in genes encoding vaccine components, such as pertussis toxin (PT), filamentous hemagglutinin (FHA), pertactin (PRN), and fimbriae (Fim2 and 3). These genetic and phenotypic changes appear to be an adaptive advantage for these strains to survive and spread among the vaccinated populations [9,10].

Iran is one of the countries using whole cell pertussis vaccine in its national vaccination program. There is a vaccination coverage of 99% for pertussis in Iran, but since 2004, there has been an increase in pertussis incidence. Whole-cell vaccine contains reference strains such as *B. pertussis* 134 and *B. pertussis* 509 [10].

Objectives: In this study, genetic variations and allele types of 4 important genes were investigated, including two regions of *prn*

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gene (*prn-1* and *prn-2*) and two serotypes of *fim* gene (*fim2* and *fim3*), in *B. pertussis* clinical strains collected from provinces of Iran and vaccine strains using polymerase chain reaction (PCR) and sequencing.

Materials and Methods

Strains collection: All suspected samples of pertussis from across the country were sent to the pertussis national reference laboratory (Pasteur Institute of Iran) during 2014-2016 years. Samples were cultured on Regan-Lowe agar medium supplemented with 20% defibrinated sheep blood and 40 µg/mL cephalexin (produced in Pasteur Institute of Iran) and incubated for 3-4 days at 35°C with humidity. Vaccine strains (*B. pertussis* 509 and *B. pertussis* 134) and reference strains (TohamaI, positive control in PCR method) were also analyzed.

Strain identification: All of the 20 *B. pertussis* isolates were confirmed by macroscopic morphological (small, shiny, and silvery colony), gram-staining, and biochemical tests (oxidase) and specific antiserum (Difco) [11].

DNA extraction: Fresh suspensions were prepared from pure and single colonies, and then genomic DNA was extracted based on the protocol of High Pure PCR Template Preparation kit (Roche Company, Germany) [12].

Polymerase chain reaction (PCR): Amplification of *fim2*, *fim3*, *prn-1,* and *prn-2* genes was done in a total volume of 25µL PCR mixture, containing 12.5µL Red Master Mix 2X containing 1.5mM Mg (Ampliqon Company, Denmark), 1µL of 10µM forward and reverse primers (Pishgam, Iran), and 2µL of template DNA. Primers information is shown in Table 1.

Sequencing: In this study, 13 out of 20 samples were randomly selected for sequencing. The samples were sent to Macrogen Research, Korea and sequenced using ABI capillary system.

Data analysis: First, the sequences quality was checked by Chromas software. Then reference genotypes of studied sequences were obtained from GenBank (NCBI). Finally, in order to determine allele types of the studied genes, clinical and vaccine strains sequences were aligned using MEGA4 software.

Findings

During 2014-2016, more than 1,000 nasopharyngeal samples of patients suspected to pertussis were sent to the Pasteur Institute of Iran. After samples cultivation and strains identification, 20 cases were positive for *B. pertussis*. These 20 samples were checked for the presence of *fim2*, *fim3*, *prn-1*, and *prn-2* genes by PCR.

Table 1) Primer information

Primer Name	Primer Sequence(5′-3′)	Target Gene	Product Size (bp)	Ta[a]	References
PRN1-F PRN1-R	GCCAATGTCACGGTCCAA GCAAGGTGATCGACAGGG	*Prn*-1	585	52	[18]
PRN2-F PRN2-R	AGCTGGGCGGTTCAAGGT CGGATTCAGGCGCAACTC	*Prn*-2	547	62	[21]
F2-F F2-R	GCGCCGGGCCCTGCATGCAC GGGGGGTTGGCGATTTCCAGTTTCTC	*Fim*2	850	67	[25]
F3-F F3-R	CACCCTCAACCATATCAA TCTTGCTGCCATTGGTGA	*Fim*3	371	53	[18]

[a] Annealing temperature

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All of the samples were also positive for those genes. Due to the study limitations, 13 cases were randomly selected and sent for sequencing (Table 2).

fim2: The sequencing results of *fim2* gene indicated that 9 (~70%) and 4 (~30%) *B. pertussis* strains under study carried *fim2-2* and *fim2-1* alleles, respectively, and vaccine strains of *Bp*134 and *Bp*509 had *fim2-1* and *fim2-2* alleles, respectively.

fim3: The sequencing results of *fim3* gene showed that 9 (~70%) and 4 (~30%) strains contained *fim3B* and *fim3A* alleles, respectively. However, both vaccine strains of *Bp*134 and *Bp*509 carried *fim3A* allele. It was evidenced that the dominant circulating strains were different from the vaccine strains in terms of the allele type.

***prn-1*:** The sequencing results of *prn* gene Region 1 showed that 10 (77%) and 3 (23%) strains carried *prn2* and *prn1* alleles, respectively. On the other hand, vaccine

Table 2. Sequenced samples information

Patients Number	Year	Age [a]	City	Vaccine History [a]
1	1393	1 y	East Azerbaijan	UNK
2	1393	7 m	Tehran	UV
3	1394	5 y	Esfahan	UTD
4	1394	1 m	Chaharmahal and Bakhtiari	UV
5	1394	3 m	East Azerbaijan	UTD
6	1394	3 y	Khorasan Razavi	UTD
7	1394	3 y	Mazandaran	UTD
8	1394	24 d	Chaharmahal and Bakhtiari	UV
9	1394	2 y	East Azerbaijan	UTD
10	1394	1 m	East Azerbaijan	UTD
11	1394	8 d	Golestan	UV
12	1395	1 m	Mazandaran	UTD
13	1395	2 y	East Azerbaijan	UTD
Tohama1	1954	-	Japan	-
Bp 134	<1950	-	USA	-
Bp 509	1950	-	The Netherlands	-

[a] Abbreviations: y, year; m, month; d, day; UTD, up to date; UNK, unknown; UV, unvaccinated

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strains of *Bp*134 and *Bp*509 had *prn1* and *prn7* alleles, respectively. It was also evidenced that the dominant circulating strains were different from the vaccine strains in terms of the allele type.

prn-2: No mutation was observed in sequencing results.

Discussion

No specific cause for whooping cough outbreak has been reported yet. The increased incidence of the disease could be due to the increased awareness of physicians and general population, improved diagnostic methods, low quality of vaccine, reduced vaccine-induced immunization after vaccination, and adaptation of *B. pertussis* bacteria [13]. It seems that the compatibility of *B. pertussis* has a significant role in its survival among the vaccinated population. One of the important causes of organism survival in the host is nucleotide changes of the bacterial genome, which occur in various forms such as removal and substitution [10,14]. Some research has been done in Iran and other countries in order to survey the polymorphism of virulence factors genes which play key roles in pertussis vaccine too. In Iran, Nikbin et al. (2015) showed that all *B. pertussis* strains had *prn2* and *fim3B* alleles [15], according to a similar study conducted on suspected cases of pertussis during 2008-2012.

In another study in Iran, Sadeghpour et al. (2018) conducted a study on suspected cases of pertussis from 2009 to early 2014. They showed that samples under study had no mutation in *prn* Region 2; all of which contained *fim2-1* allele for *fim2* gene [16].

According to the results of the present study conducted during 2014-2016, the dominant alleles of the pertactin Region 1 and *fim3* genes were similar to those of the above studies in Iran; however, 23% of the samples had *prn1* allele, and 30% of the samples had *fim3A* allele, while in the Nikbin et al.'s study, these alleles were not present in the circulating strains. On the other hand, in the present study, the dominant allele for *fim2* gene was *fim2-2,* and only 30% of the samples had *fim2-1* allele, while in the Sadeghpour et al.'s study, all samples had *fim2-1* allele. According to the present study results about the vaccine strains polymorphism in Iran, it was determined that both vaccine strains of Bp134 and Bp509 had *fim2-1/fim3A/prn1* and *fim2-2/fim3A/prn7* alleles, which were significantly different from the polymorphism results of the circulating strains in aforementioned studies. Actually, there is a difference regarding the dominant allele between the circulating and vaccine strains.

In a study in Great Britain during 1920-2002, surveying the mutations in *fim2* gene, it was shown that 75% of the *B. pertussis* strains contained *fim2-1* allele, and the rest of the strains carried *fim2-2* allele [17].

In a study conducted during 1949-1999, 196 samples collected from 5 countries were analyzed. It was found that 41, 34, and 22% of the strains had *prn2*, *prn1,* and *prn3* alleles, respectively, and the rest (less than 2%) carried *prn4, 5,* and *6* alleles. Also, 84 and 16% of the strains had *fim2-1* and *fim2-2* alleles, respectively [18].

In France in a research conducted on the polymorphism of *B. pertussis* positive samples during 1991-2001, it was determined that most of the studied strains carried *prn2* allele [19].

In a comprehensive study conducted from 1998 to 2001, 201 *B. pertussis* positive samples collected from five European countries were analyzed. The results showed that the predominant pertactin gene allele in all the studied countries was *prn2*, ranging from 75% in France to 95% in the Netherlands. In their study, there were only 3 and 10 samples with *prn1* and *prn3* alleles,

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respectively. However, vaccine strains used for whooping cough in the studied countries carried *prn1* allele [20].

In a similar study conducted in Argentina during 1997-2003, it was shown that *prn2* was the dominant circulating allele, and *prn1* allele was ranked at a much lower rate [21].

In a study in Canada, 52 *B. pertussis* strains isolated from patients during 2002-2014 were analyzed, and it was found that more than 65% of the samples (most of them) carried *prn2* and *fim3B* alleles [22].

In a polymorphism study conducted on 33 *B. pertussis* clinical isolates from California and Vermont during 2010-2012, coinciding with the outbreak of pertussis in these two cities, it was shown that all the isolated strains, except for one case, had *prn2* allele. Also, about 70% of the strains had *fim3A*, and the rest contained *fim3B* alleles. Meanwhile, polymorphism of the two vaccine strains of C393 and E476 showed that both strains had *prn1* and *fim3A* alleles [23].

In the United States, the polymorphism study of *B. pertussis* strains until 2012 showed that most of the strains had *prn2* allele [24].

Comparing the results of the previous and present studies conducted in Iran, it was found that except for *prn2* and *fim3B*, which were the dominant circulating alleles, only *fim2* had an allelic change because in the previous studies, *fim2-1* and in the present study, *fim2-2* were identified as the dominant alleles. However, vaccine strains had no *prn2* and *fim3B* alleles, while they were identified as the dominant circulating alleles.

Comparing the results of the present and other studies conducted in different countries, it seems that there was a change in dominant circulating allele from *prn1* to *prn2* in pertactin gene, while most produced vaccines have *prn1* allele. Regarding *fim2* gene, apparently, most of the circulating strains in most countries have *fim2-1* allele, while in the present study, *fim2-2* allele was reported as 70%. Regarding *fim3* gene, it seems that in other countries, *fim3B* is also the dominant allele.

Conclusion

Finally, evidence suggests that circulating strains had allelic changes in their pathogenic genes. According to the research conducted in Iran in the last decade and due to the fact that vaccine strains do not contain *fim3B* and *prn2* alleles (alleles present in dominant circulating strains), it is suggested that the efficacy of commercial vaccines should be increased by using strains more similar to the circulating strains in the manufacture protocol. There is no doubt that more research is needed in this area.

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