Sequence Variation of the Pertussis Toxin S1 Subunit Encoding Gene in the Clinical Isolates of *Bordetella pertussis* in Iran

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1. Background

Pertussis infection in humans is caused by the bacterium *Bordetella pertussis* (*B. pertussis*). According to Nils Rosen von Rosenstein, a Swedish doctor, the disease first emerged in France in 1414. The first outbreak of the disease was described in 1578 by Guillaume de Baillou book (1). In the twentieth century, pertussis was one of the most common childhood diseases and a major cause of death in children in America. Before vaccination, pertussis was one of the major causes of death. The whole-cell vaccine, which was released in 1950, significantly reduced the disease. Since 1970, the number of cases of whooping cough has been on the increase, possibly due to changes in case definition, reduction of vaccination coverage, suboptimal vaccines, waning vaccine-induced immunity and most important of all, pathogen adaptation and genetic variation (3). However, according to the centers for disease control and prevention (CDC), a large number of cases of the disease are unknown and unreported (2).

*B. pertussis* possesses a number of virulence factors which play an important role in its pathogenesis. Pertussis toxin is one of the most important virulence factors. It is an AB5 exotoxin and is recognized as one of the most complex bacterial toxins. The toxin is composed of the A (S1) subunit, which is a single polypeptide, and the B oligomer, which is composed of 5 polypeptides. The A subunit has catalytic, enzymatic, and toxic activities, while the B oligomer contains the domains of binding-receptors and is responsible for entry into host cells. The A subunit of pertussis toxin is the most immunogenic and polymorphic subunit (4).

Small genetic changes such as mutations can have remarkable consequences on public health. In the era before vaccination, *pertussis* was one of the major causes of death. The whole-cell vaccine, which was released in 1950, significantly reduced the disease. Since 1970, the number of the cases of whooping cough has been on the increase, however. It seems that the bacterium over time has made changes to the genes of its virulence factors and, as such, gained the ability to adapt to the new environment (3). Thus far, totally 8 alleles have been identified for the *pertussis toxin S1 subunit* (*ptxS1*) gene (Figure 1) (4).
Figure 1. Eight Alleles of pertussis toxin subunit 1 (ptxA), Based on Amino Acid Sequences

| ptxA1 | ptxA2 | ptxA3 | ptxA4 | ptxA5 | ptxA6 | ptxA7 | ptxA8 |
|-------|-------|-------|-------|-------|-------|-------|-------|
| 38    | 68    | 85    | 129   | 196   | 228   | 232   | 238   |

Vaccination with the whole-cell vaccine began in 1949 in Iran, with the vaccine recommended at ages 2, 4, 6, and 18 months of age (5). The commencement of the vaccination initially reduced the incidence of pertussis; nevertheless, the past few years have seen a rise in the prevalence of pertussis. In Iran, similar to other countries, whooping cough has also been observed in adults like children, who are less secure.

Several studies have probed into the genetic diversity of the B. pertussis virulence factors and its immunogenicity. Since pertussis toxin is considered the most polymorphic and immunogenic virulence factor, we studied the polymorphisms of the encoding genes of the catalytic portion of the toxin (i.e. ptxS1).

2. Objectives

The aim of this research was to determine the alleles of ptxS1 in the circulating strains of B. pertussis and compare them to the vaccine strain.

3. Patients and Methods

3.1. Sample Collection and Isolation of Bordetella pertussis

Samples were taken from suspected patients with whooping cough in health centers across the country using nasopharyngeal Dacron swabs. The swab samples were transferred to the Pertussis Reference Laboratory at Pasteur Institute of Iran.

The samples were cultured on a specific medium, Regan-Lowe and Bordet-Gengou, containing 10% defibrinated sheep blood with cephalaxin (40 μg/mL) (Sigma Chemical Co., USA). Biochemical tests on the suspected Gram-negative coccobacilli were performed using the API20E Kit (BioMérieux SA, France). The B. pertussis antisera (Difco, BBL) was used for the final approval by the slide agglutination test. In this study, 50 B. pertussis isolated strains were studied: 9 strains of 2009 - 2011 and 41 strains of 2012 - 2013. The DNA extraction of the studied strains was performed using the high pure PCR template preparation kit (Roche applied science).

3.2. Polymerase Chain Reaction and DNA Sequencing

A polymerase chain reaction (PCR) mixture, containing 12.5 μL of Taq polymerase 2 x master mix red (Ampliqon ApS, Denmark) and 5 μM of each primer, was added to a final volume of 25 μL. Amplification was performed with the following conditions: pre denaturation for 10 minutes at 94°C; 30 cycles of denaturation for 2 min at 94°C; annealing for 2 minutes at 60°C; extension for 1 minute at 72°C; and a step of the ultimate extension for 10 minutes at 72°C.

For pertussis toxin, primers of S1-F2 (5'-CCCTGCGATTGTAAGTCGATC-3') and S1-R2 (5'-AGAGCGCATTCCTGGTCGATC-3') were used to amplify a 934 bp product that contained the complete ptxS1 gene (Figure 2) (6).

Sequencing was performed using the ABI capillary system (Macrogen Research Seoul, Korea). Strain 134, which is used for whole-cell pertussis vaccine in Iran, was also studied.

4. Results

Fifty clinical strains of B. pertussis were adjusted based on the date of isolation and age of the patients. Children in the age range of 2 - 18 months constituted the dominant group (Table 1).

Figure 2. Gel Electrophoresis of the Bordetella pertussis Clinical Isolates

M is the marker, and No. 1 to No. 4 are related to B. pertussis clinical isolates, and No. 9 and No. 10 are positive and negative controls, respectively.
In this study, the molecular weight of the PCR product of the ptxS1 (pta) gene was 934 bp (Figure 2). The PCR results showed that all the strains had the ptxS1 gene. The standard strain of B. pertussis ATCC 9797 was used as a positive control.

4.1. Sequencing of pertussis Toxin S1 Subunit (ptxS1) Allele of Bordetella pertussis Clinical Isolates

The sequence analysis of the samples was done using Chromas and Mega 4. All the isolates showed a ptxS1A dominant allele and the other alleles were not observed (Table 1). The strain of B. pertussis 134 was studied as the vaccine strain and showed a ptxS1B allele.

5. Discussion

B. pertussis is the causative agent of whooping cough, which is known as an acute respiratory disease among children worldwide. The best way to prevent the illness is vaccination among infants, children, adolescents, and adults (7).

Pertussis vaccine is prepared in either whole-cell vaccine or acellular vaccine in the world. In Iran, the whole-cell vaccine has been used so far (8).

Although pertussis surveillance and vaccination coverage have been augmented in recent years, pertussis is on the increase in many European and American countries. Reduced efficacy of vaccines, improved laboratory diagnosis, reduced immunity due to vaccination, and allelic shift to new alleles may be the most important reasons for the pertussis resurgence (9, 10).

The present investigation is the first study on the gene diversity of pertussis toxin, as one of the most important virulence factors of B. pertussis, to be conducted in Iran. This study was performed on clinical strains and vaccine strain 134. The sequencing results of the ptxS1 gene of the pertussis toxin of the vaccine strain showed that strain 134 possessed the ptxS1B allele. The results of the clinical strains demonstrated that children aged less than 2 years constituted the main age group of the patients and all the tested strains (100% strains) exhibited the ptxS1A allele. Unfortunately, there is no information on the strains of the pre-vaccination era in Iran. However, our results showed an allelic shift towards the ptxS1A allele, which is a different allele from the vaccine strain used in the country. Strategies for vaccine preparation in our country should be established based on isolated strains from patients.

The first study on the long-term effects of vaccination (about 44 years) was performed in the Netherlands by Mooi et al. who probed into the strain polymorphisms in the circulating isolates and observed that the ptxS1 gene was polymorphic and its alleles showed changes over time. The frequent observation by several studies that B. pertussis strains do not respond to commonly used vaccines and continue to circulate in society highlights the allelic shifts in B. pertussis (11). Moreover, previous investigations have identified the ptxS1A, ptxS1B, or ptxS1B alleles in older strains or vaccinal strains and the ptxS1A allele in the circulating strains. These allelic changes have been reported in many European, American, and Asian strains (12-15).

The whole-cell vaccine was introduced in the Netherlands in the early 1950s; and in the course, all the strains had the ptxS1B allele in the vaccine strain. There was an antigenic shift towards the ptxS1A allele in subsequent years, however (4, 11).

Samples collected in Denmark in 1949 - 2010 showed that the B. pertussis population had continued to exhibit a dominant ptxS1A allele (16). Similar observations of allelic change have been reported in American and European countries, including England, Finland, France, Canada, Russia, Argentina, America, Germany, and Poland (17-26).

A study by van Gent et al. reported an allelic variation in the pertussis toxin A subunit encoding gene (ptxA) (27). In a study conducted by Nikbin et al. in Queensland, all of the tested samples had the ptxS1A allele (28). Another study was performed on the allelic diversity of the pertussis toxin gene by Fry et al. who reported that ptxA1 had the most allelic diversity (21).

Investigations carried out in France over the past 10 years have shown that B. pertussis circulating strains have expressed ptxA1 against the vaccine strain since 1991 (2). Petersen et al. examined the ptxA genes during three time periods. In the first period, the 3 alleles of ptxA1 (13%), ptxA2 (35%), and ptxA4 (52%) were identified. In the second period, ptxA4 was not detected but ptxA2 and ptxA1 rose to 11% and to 89%, correspondingly. In the third period, ptxA1 was the only allele that was identified (16).

According to the vaccination history of Iran, this country can be classified among the countries with high vaccination coverage, which begs the question as to why there has been a re-emergence of pertussis. Are the circulating pertussis strains consistent with vaccine-mediated immunity? What factors have led to the re-emergence of pertussis?
sis? Probably in Iran, incomplete vaccination of individuals, genetic changes in the strains, and possible differences between the circulating strains and the strain used in the vaccine and also carrier adults (due to loss of immunity to this bacterium over time) constitute the significant causes of this disease in the vaccinated individuals (29).

Despite vaccination programs, similar allelic shifts in different countries are seen as well. In other words, the ptxS1A allele, which is different from the allele present in the vaccine strain, is the dominant allele circulating in different countries. This indicates that relatively common alleles are observed in the vaccine strains used throughout the world, and these antigenic changes have occurred in order to enable the microorganism to evade the human immune response and survive (3). Indeed, it appears that allelic shifts have enabled B. pertussis strains to escape host immunity and continue to survive and cause infection.

Molecular and immunological evidence suggests that immune selection has an effect on changes in the S1 subunit. Mutations are confined to the molecular regions in the S1 subunit, which have been identified as a T-cell and B-cell epitope, such that the B and T cells lose the ability to identify microorganisms. Our results, obtained from Iranian strains isolated from patients with pertussis, are similar to those obtained from many countries boasting high vaccination coverage. In light of the information from Iran and European and American countries on the dominant ptxS1A allele in recent decades, this change can be deemed a global phenomenon (4).

The other important virulence factors of B. pertussis are pertactin, fimbriae, adenylate cyclase toxin, tracheal cytotoxin, and filamentous hemagglutinin, which should be investigated in further studies on the polymorphisms and dominant alleles of their encoding genes.

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Authors’ Contributions

All authors read and approved the submission of this manuscript and contributed to this research.

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