

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

Maryam Hosseinpour¹; Nazanin Jannesar Ahmadi¹; Vajiheh Sadat Nikbin¹; Fereshteh Shahcheraghi^{1,*}

¹Pertussis Reference Laboratory, Department of Bacteriology, Pasteur Institute, Tehran, IR Iran

*Corresponding author: Fereshteh Shahcheraghi, Pertussis Reference Laboratory, Department of Bacteriology, Pasteur Institute, Tehran, IR Iran. Tel/Fax: +98-2166405535, E-mail: shahcheraghifereshteh@yahoo.com

Received: June 21, 2014; Revised: June 6, 2015; Accepted: June 14, 2015

Background: Whooping cough (pertussis) is an acute respiratory disease caused by *Bordetella pertussis* (*B. pertussis*). Pertussis toxin is an important virulence factor of *B. pertussis* and plays a major role in the immune and inflammatory responses. Likewise, allelic variations in the genes of virulence factors have led to the non-responsiveness of the new strains to both whole-cell and acellular vaccines. Given the importance of pertussis vaccine, we sought to address the lack of fundamental studies on the polymorphisms of the virulence genes of *B. pertussis* in Iran.

Objectives: The aim of this study was to identify the polymorphisms of the *pertussis toxin S1 subunit (ptxS1)* gene in the circulating strains and compare them to the vaccine strain.

Patients and Methods: In this study, 50 strains of *B. pertussis* isolated from patients with pertussis were investigated in the pertussis reference laboratory of Pasteur institute of Iran. Cultivation, biochemical tests, and the specific antisera were used to confirm *B. pertussis*. The sequencing of the polymerase chain reaction products was performed to determine the *ptxS1* alleles, and *B. pertussis* 134 was studied as the vaccine strain.

Results: The results showed that all the strains had the dominant allele *ptxS1A*. There were differences between the alleles of the clinical strains and the vaccine strain.

Conclusions: In recent years, a significant increase in the incidence of pertussis has been reported worldwide. Our findings regarding the allelic shift of the *ptxS1* gene are similar to those reported in many European and American countries showing the difference of the dominant allele of *ptxS1* between the circulating isolates and the vaccine strains.

Keywords: Pertussis Vaccine; Whooping Cough; *Bordetella pertussis*

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 the availability of pertussis vaccine in the 1940s, more than 200,000 cases of the disease were reported annually (2).

Various factors have been suggested for the resurgence of pertussis such as increased awareness, improved diagnosis, 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

	38	68	85	129	196	228	232	238
ptxA1	DPPAT	VLDHL	AFVST	ADNNF	EYNSA	LVRIAPVIGAC		MARQA
ptxA2M.....	
ptxA3
ptxA4E..M..V..	
ptxA5E..P..	..M..M..	
ptxA6
ptxA7
ptxA8S..M.....	

Dots show the same amino acids (4).

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 cephalexin (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* anti-serum (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

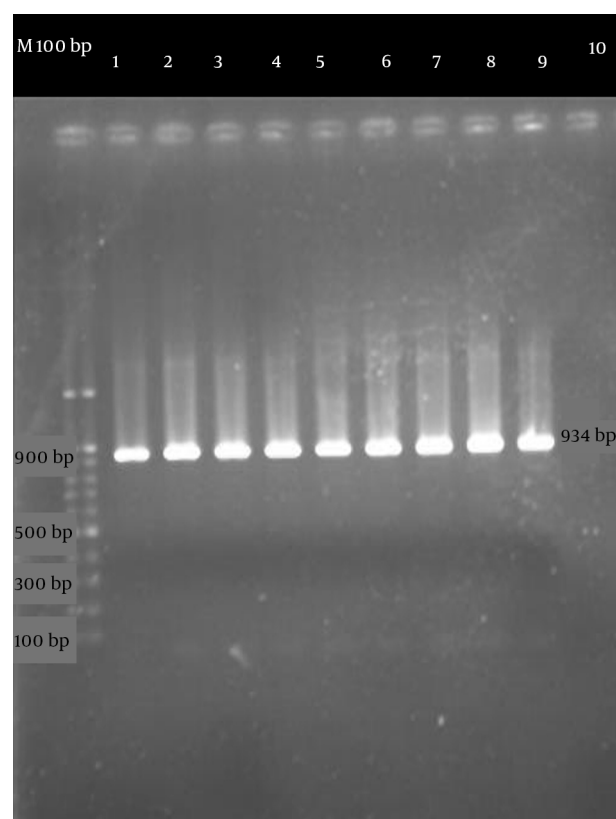
A polymerase chain reaction (PCR) mixture, containing 12.5 µL of Taq polymerase 2 x master mix red (Ampliqon ApS, Denmark) and 5 µM of each primer, was added to a final volume of 25 µL. Amplification was performed with the following conditions: pre denaturation for 10 minutes at 94°C; 30 cycles of denaturation 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'-CCCCTGCCATG-GTGTGATC-3') and S1-R2 (5'-AGAGCGTCTTGC GGTCGATC-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. 8 are related to *B. pertussis* clinical isolates, and No. 9 and No. 10 are positive and negative controls, respectively.

Table 1. *Bordetella pertussis* Isolates Based on the Time of Isolation and Age of the Patients

Allele	Above 10 Years	18 Months -10 Years	2 Months-18 Months	Under 2 Months	Isolates Per Year (No.)	Year of Isolation
<i>ptxS1A</i>	1	2	4	2	9	2009 - 2011
<i>ptxS1A</i>	2	2	12	7	23	2012
<i>ptxS1A</i>	-	1	9	8	18	2013
<i>ptxS1A</i>	3	5	25	17	50	Total isolates

In this study, the molecular weight of the PCR product of the *ptxS1* (*ptxA*) 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 vaccinal 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 *ptxS1D*, *ptxS1B*, or *ptxS1E* 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* or *ptxS1D* 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 pertus-

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.

Acknowledgements

We thank the staff of the microbiology department of Pasteur institute of Iran, the department of communicable disease management center of the Iranian ministry of health and medical education, and Razi Vaccine and serum research center.

Authors' Contributions

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

Financial Disclosure

Pasteur Institute of Iran has covered all the costs of the project.

Funding/Support

Doctor Shahcheraghi, Head of the bacteriology department of Pasteur institute of Iran, was responsible for the financial support of this project.

References

- Lapin JH. *Whooping Cough*, Springfield, Ill. Charles C Thomas; 1943.
- Peleg AY, Hooper DC. Hospital-Acquired Infections Due to Gram-Negative Bacteria. *N Engl J Med*. 2010;**362**(19):1804-13.
- Mooi FR, Van Der Maas NA, De Melker HE. Pertussis resurgence: waning immunity and pathogen adaptation - two sides of the same coin. *Epidemiol Infect*. 2014;**142**(4):685-94.
- Mooi FR. Bordetella pertussis and vaccination: the persistence of a genetically monomorphic pathogen. *Infect Genet Evol*. 2010;**10**(1):36-49.
- Zarei S, Tehrani MJ, Akhouni M, Zarnani A. [Early complications of diphtheria tetanus pertussis vaccine triple the number of 4-6 year old children in Tehran]. *Payesh*. 2007;**6**(3):273-83.
- Bottero D, Gaillard ME, Fingerhann M, Weltman G, Fernandez J, Sisti F, et al. Pulsed-field gel electrophoresis, pertactin, pertussis toxin S1 subunit polymorphisms, and surfaceome analysis of vaccine and clinical Bordetella pertussis strains. *Clin Vaccine Immunol*. 2007;**14**(11):1490-8.
- Centers for Disease C, Prevention. Updated recommendations for use of tetanus toxoid, reduced diphtheria toxoid, and acellular pertussis (Tdap) vaccine in adults aged 65 years and older - Advisory Committee on Immunization Practices (ACIP), 2012. *MMWR Morb Mortal Wkly Rep*. 2012;**61**(25):468-70.
- Zhang L, Prietsch SO, Axelsson I, Halperin SA. Acellular vaccines for preventing whooping cough in children. *Cochrane Database Syst Rev*. 2012;**3**:CD001478.
- De Serres G, Boulianne N, Douville Fradet M, Duval B. Pertussis in Quebec: ongoing epidemic since the late 1980s. *Can Commun Dis Rep*. 1995;**21**(5):45-8.
- Bass JW, Wittler RR. Return of epidemic pertussis in the United States. *Pediatr Infect Dis J*. 1994;**13**(5):343-5.
- Mooi FR, van Oirschot H, Heuvelman K, van der Heide HG, Gaastra W, Willems RJ. Polymorphism in the Bordetella pertussis virulence factors P.69/pertactin and pertussis toxin in The Netherlands: temporal trends and evidence for vaccine-driven evolution. *Infect Immun*. 1998;**66**(2):670-5.
- Yao SM, Lin YC, Chou CY, Chen YY, Hsiao MJ, Chen HY, et al. Antigenic divergence of Bordetella pertussis isolates in Taiwan. *J Clin Microbiol*. 2005;**43**(11):5457-61.
- King AJ, van Gorkom T, Pennings JL, van der Heide HG, He Q, Diavatopoulos D, et al. Comparative genomic profiling of Dutch clinical Bordetella pertussis isolates using DNA microarrays: identification of genes absent from epidemic strains. *BMC Genomics*. 2008;**9**:311.
- De Schutter I, Malfroot A, Dab I, Hoebrex N, Muyldermans G, Pierard D, et al. Molecular typing of Bordetella pertussis isolates recovered from Belgian children and their household members. *Clin Infect Dis*. 2003;**36**(11):1391-6.
- Byrne S, Slack AT. Analysis of Bordetella pertussis pertactin and pertussis toxin types from Queensland, Australia, 1999-2003. *BMC Infect Dis*. 2006;**6**:53.
- Petersen RF, Dalby T, Dragsted DM, Mooi F, Lambertsen L. Temporal trends in Bordetella pertussis populations, Denmark, 1949-2010. *Emerg Infect Dis*. 2012;**18**(5):767-74.
- Borisova O, Kombarova SY, Zakharova NS, van Gent M, Aleshkin VA, Mazurova I, et al. Antigenic divergence between Bordetella pertussis clinical isolates from Moscow, Russia, and vaccine strains. *Clin Vaccine Immunol*. 2007;**14**(3):234-8.
- Cassiday P, Sanden G, Heuvelman K, Mooi F, Bisgard KM, Popovic T. Polymorphism in Bordetella pertussis pertactin and pertussis toxin virulence factors in the United States, 1935-1999. *J Infect Dis*. 2000;**182**(5):1402-8.
- Elomaa A, Advani A, Donnelly D, Antila M, Mertsola J, Hallander H, et al. Strain variation among Bordetella pertussis isolates in Finland, where the whole-cell pertussis vaccine has been used for 50 years. *J Clin Microbiol*. 2005;**43**(8):3681-7.
- Fingerhann M, Fernandez J, Sisti F, Rodriguez ME, Gatti B, Bottero D, et al. Differences of circulating Bordetella pertussis population in Argentina from the strain used in vaccine production. *Vaccine*. 2006;**24**(17):3513-21.
- Fry NK, Neal S, Harrison TG, Miller E, Matthews R, George RC. Genotypic variation in the Bordetella pertussis virulence factors

- pertactin and pertussis toxin in historical and recent clinical isolates in the United Kingdom. *Infect Immun*. 2001;**69**(9):5520-8.
22. Gzyl A, Augustynowicz E, Rabczenko D, Gniadek G, Slusarczyk J. Pertussis in Poland. *Int J Epidemiol*. 2004;**33**(2):358-65.
 23. Hopkins RS, Jajosky RA, Hall PA, Adams DA, Connor FJ, Sharp P, et al. Summary of notifiable diseases—United States, 2003. *MMWR Morb Mortal Wkly Rep*. 2005;**52**(54):1-85.
 24. Litt DJ, Neal SE, Fry NK. Changes in genetic diversity of the *Bordetella pertussis* population in the United Kingdom between 1920 and 2006 reflect vaccination coverage and emergence of a single dominant clonal type. *J Clin Microbiol*. 2009;**47**(3):680-8.
 25. Njamkepo E, Rimlinger F, Thiberge S, Guiso N. Thirty-five years' experience with the whole-cell pertussis vaccine in France: vaccine strains analysis and immunogenicity. *Vaccine*. 2002;**20**(9-10):1290-4.
 26. Skowronski DM, De Serres G, MacDonald D, Wu W, Shaw C, Macnabb J, et al. The changing age and seasonal profile of pertussis in Canada. *J Infect Dis*. 2002;**185**(10):1448-53.
 27. van Gent M, Bart MJ, van der Heide HG, Heuvelman KJ, Mooi FR. Small mutations in *Bordetella pertussis* are associated with selective sweeps. *PLoS One*. 2012;**7**(9):e46407.
 28. Nikbin VS, Shahcheraghi F, Lotfi MN, Zahraei SM, Parzadeh M. Comparison of culture and real-time PCR for detection of *Bordetella pertussis* isolated from patients in Iran. *Iran J Microbiol*. 2013;**5**(3):209-14.
 29. Shahcheraghi F LMPMNVSFZS. Isolation of *Bordetella pertussis* and parapertussis from clinical samples collected from all over Iran. *Mazandaran Univ Med Sci*. 2012;**22**(88):2-8.