Polymorphism in *Bordetella Pertussis* Virulence Factors in Serbia before Switch from Whole Cell to a Cellular *Pertussis* Vaccine

**Abstract**

**Background:** Mass vaccination has significantly reduced the incidence of *pertussis*; however, the disease is re-emerging even in some countries with high vaccination coverage. In Serbia, whole cell *pertussis* vaccine was introduced in 1957 and it has been used since 2014.

**Methods:** To monitor changes in bacterial population, 77 isolates collected from 1953 to 2013 were studied. The methods included serotyping of fimbriae (Fim), genotyping of pertactin (prn), *pertussis* toxin S1 subunit (ptxA) and pulsed-field gel electrophoresis analysis (PFGE).

**Results:** Shift from ptxA2 to ptxA1 has been observed in isolates since the late of 1960s. In the period 1961-1979 genotype ptxA1 became as common as genotype ptxA2. After that, during the period of 1980-1989, the predominant ptx genotype was ptxA1. Re-appearance of the ptxA2 allele followed an addition of the two strains harboring ptxA1 in the vaccine in 1985. The allele prn1 was predominant among the Serbian isolates, though prn3 and prn11 have been detected since 1981. The prn2 allele was only found in one strain isolated in 1984, two of the four strains isolated in 2000 and three strains from 2011. Serotype Fim2.3 disappeared before 1980 and serotype Fim2 became predominant since then. The four vaccine strains represented four PFGE profiles. Twenty-two (40%) isolates tested by PFGE produced 22 distinct profiles. Twenty-four (43%) isolates had unique Serbian profiles (BpSBR).

**Conclusion:** The results of this present study indicate that the *B. pertussis* population in Serbia is different from other vaccinated populations and that this difference may be related to the vaccine used for 57 years.

**Keywords:** *Bordetella pertussis*; *Pertussis* toxin; Pertactin; PFGE; Serotyping; Fimbriae; Electrophoresis

**Abbreviations:** Fim: Fimbriae; prn: Pertactin; PFGE: Pulsed-Field Gel Electrophoresis Analysis; DTPw: Diphtheria-Tetanus-Whole Cell *Pertussis*; ptxA: *Pertussis* toxin subunit

**Introduction**

*Pertussis* (whooping cough) is a worldwide infectious disease caused by the bacteria *Bordetella pertussis* [1]. Despite the fact that the introduction of vaccination in the 1950s and 1960s has reduced *pertussis* morbidity and mortality, this disease is still prevalent [2] and *pertussis* is still one of the leading causes of vaccine preventable deaths in the world [3]. *Pertussis* is a respiratory tract infection transmitted by aerial droplets with an incubation period of 7-10 days, which remains contagious for up to 3 weeks after the appearance of the first signs if no treatment is given. Mass vaccination has significantly reduced the incidence of *pertussis*; however, the disease is re-emerging even in some countries with high vaccination coverage. The resurgence of *pertussis* in countries, such as the Netherlands, the United States, Canada and Australia, has been studied to find an explanation for its re-emergence [2,4-7]. Moreover, in these countries antigenic divergence with respect to *pertussis* toxin (Ptx) and pertactin (Prn) has been found between *Bordetella pertussis* vaccine strains and clinical isolates. In Serbia vaccination against *pertussis* has been from 1957. The diphtheria-tetanus-whole cell *pertussis* (DTPw) vaccine has been manufactured in the Institute of Virology, Vaccine and Sera Torlak, Belgrade, Serbia. The DTPw vaccine was given at 2, 4, 6, and 12 months of age. A second booster dose with the mono *pertussis* vaccine was given at 4 years of age during the periods from 1970 to 1981 and from 1990 to 2000. Last composition of the whole-cell vaccine has been used since 1985 and contained four *B. pertussis* strains. The vaccine strains were chosen in compliance with serotype, immunogenicity and specific toxicity. The four strains represented three serotypes: Fim2 (8/84), Fim2,3 (1772/57 and 2047/57) and Fim3 (23/81). The vaccine strains 2047/57 and 1772/57 represented ptxA2/prn1 genotypes, whereas the vaccine strains 23/81 and 8/84 harbor ptxA1/prn1 and ptxA1/prn2. All vaccines strains were...
in equal amount in the vaccine composition [8]. The reported vaccination coverage in Serbia ranged from 79% to 98% (median, 90%) in 1981-2013 [3]. In Serbia, the whole-cell pertussis vaccine has been replaced with a cellular vaccine in 2014. The aim of this study was to analyze B. pertussis isolates circulating between 1953 and 2013 in Serbia by standard typing methods [9] and to compare them with those circulating in other European countries, USA and Australia.

Materials and Methods

B. pertussis strains and patient information

This study included 77 B. pertussis isolates. Detailed information on each of vaccine strains and clinical isolates was included in the Supplementary data. Clinical isolates were selected from the B. pertussis strain collection of the Institute of Virology, Vaccine and Sera Torlak, Belgrade, Serbia. Information on age, gender and vaccination was available in 64 patients. Their age ranged from 2 months to 33 years (mean, 6.06 years; median, 5 years). Bacteria were grown at 36°C for 72h on Bordet Gengou agar supplemented with 30% defibrinated sheep blood and subcultured on the same medium for 24h [10].

Fimbriae serotyping of B. pertussis isolates

Serotyping was performed with monoclonal antibodies against Fim2 and Fim3 by slide agglutination test [9,11].

Genotyping of Ptx S1 subunit (ptxA) and prn

The standardized genotyping of Ptx S1 subunit (ptxA) and prn was performed by sequencing and Light Cycler PCR [11-13]. These methods have been recommended for the epidemiological typing of B. pertussis isolates [9].

PFGE analysis

For the PFGE analysis, six international reference strains were included [9] and nomenclature was based on the defined profiles already observed in Finland (BpFINR) and Sweden (BpSR) [11,12]. Profiles assigned BpSBR have been found only among the Serbian isolates analyzed.

Statistical analysis

A chi-square test was used to compare frequencies of strain genotypes and serotypes between four time-periods (1953-1960, 1961-1979, 1980-1989 and 1990-2013). A p-value ≤0.05 was considered as statistically significant. Selection of time-periods was performed according to the epidemiological data.

Results

Epidemiology of pertussis in Serbia

In Serbia, pertussis is a notifiable infectious disease, which is collected in the Infectious Disease Register of the National Public Health Institute. The incidence of pertussis in Serbia has been decreasing since the introduction of vaccination at the end of the 1950s and beginning of the 1960s (Figure1). All isolates prior to 1960 were recovered from unvaccinated patients. In the period 1980-1989, 82.35% of patients were vaccinated while there were 64.3% of vaccinated patients in the period 1990-2013. There were no epidemiological data for strains isolated from patients in period 1960-1979.

Fimbriae serotyping

All three serotypes, Fim2, Fim2.3, and Fim3, were observed among the clinical isolates. The frequency of each serotype has changed over time. Before the introduction of vaccination, the prevalent serotypes were Fim 2(38%) and Fim 2.3(62%). After the introduction of vaccination, the frequency of serotype Fim2.3 decreased, being significantly lower, 0% in 1980-1989 and 23% in 1990-2013, compared to those observed in 1953-1960 and 1961-1979 (P = 0.00013 and 0.0003) (Table 1). Although the serotype Fim3 started to appear, Fim2 has been the most prevalent serotype during the study period (Figure 2).

Genotypes of ptxA and prn

All strains isolated from 1953 to 1960 were ptxA2 genotype. Shift from ptxA2 to ptxA1 has been observed in isolates since the late of 1960s. In the period 1961-1979 genotype ptxA1 became as common as genotype ptxA2. After that, during the period of 1980-1989, the predominant ptx genotype was ptxA1 (91.2%). Re-appearance of the isolates containing ptxA2 was noticed after...
the two strains harboring ptxA1 were added into the vaccine in 1985 [8]. During the period of 1990-2013, both ptxA genotypes were present in the population (Figure 3).

In the first two observed periods, 1953-1960 and 1961-1979, all isolated strains were prn1 genotype. Although the allele prn1 was mostly predominant among the Serbian isolates analyzed, prn2, prn3 and prn11 occurred in some isolates since 1980s and finally prn2 genotype became predominant in the period 1990-2011. The allele prn3 and prn11 were first detected in 1981 and 1984, respectively, and became more frequent in 1990-2013. The prn2 allele was only found in one strain isolated in 1984, two of the four strains isolated in 2000 and three strains from 2011. The frequency of prn1 in 1953-1960 and 1961-1979 was significantly higher than those observed in 1980-1989 and 1990-2013 (P <0.001 in both groups) (Table 1 & Figure 4). The frequency of prn11 in 1980-1989 and 1990-2013 was significantly higher than that found in 1953-1960 and 1961-1979 (P <0.001 in both groups).

All the four isolates harboring prn3 contained ptxA1, whereas five of the six isolates harboring prn11 represented ptxA2. All the six isolates harboring prn11 were serotype Fim2, whereas three of the four isolates harboring prn3 were serotype Fim3.

Table 1: Temporal trends in serotypes and genotypes of pertussis toxin and pertactin in Serbia.

| Year of Isolation | No of Isolates | ptxA (no) | prn (no) | serotype (no) |
|-------------------|---------------|----------|----------|--------------|
|                   |               | ptxA1    | ptxA2    | prn1 | prn2 | prn3 | prn11 | Fim2 | Fim2,3 | Fim3 |
| 1953-1960         | 21            | 0        | 21       | 21   | 0    | 0    | 0     | 8    | 13    | 0    |
| 1961-1979         | 9             | 4        | 5        | 9    | 0    | 0    | 0     | 0    | 6     | 3    |
| 1980-1989         | 34            | 31       | 3        | 27   | 1    | 3    | 3     | 22   | 0     | 12   |
| 1990-2014         | 13            | 10       | 3        | 4    | 5    | 1    | 3     | 7    | 3     | 3    |
| Total             | 77            | 45       | 32       | 61   | 6    | 4    | 6     | 37   | 22    | 18   |

PFGE profiles

The four vaccine strains represented four PFGE profiles (Figure 5). The 56 isolates tested by PFGE produced 22 distinct profiles (Figure 6). The five common profiles represented about two thirds of isolates (17 isolates with BpSR23, eight with BpFINR1, five with BpFINR9, four with BpSBR6 and three with BpSBR5). Twenty-four (43%) isolates had unique Serbian profiles (BpSBR). Change in PFGE profiles was observed over time. All PFGE profiles, except BpSR23, observed in 1950s disappeared since then. The profile BpSR23 was found in all the study periods. Of the 56 isolates tested by PFGE, 53 (95%) belonged to two clusters, having a high similarity with a minimum of 78% overall relatedness (Figure 6). Of the six isolates harboring prn11, four had the profile BpSBR6, one had BpSBR7, and one had BpSBR14. They all fell into the same cluster. Of the four isolates harboring
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Discussion

Vaccinations with Pw vaccines were introduced in the 1940s to 1960s and have successfully reduced morbidity and mortality of pertussis throughout the world [14]. Pw vaccines have been produced by different manufacturers, and the B. pertussis vaccine strains vary. The vaccine strains, both for whole-cell and cellular vaccines were usually isolated in the 1940s to 1960s, and in many countries the vaccinations have selected circulating isolates dissimilar to the vaccine strains [4,12,15,16]. Further, resurgence of pertussis has been observed in countries with long term pertussis vaccination [7,16-19]. In Serbia Pw vaccine has been in use from 1957 to 2014, and was manufactured in the Institute of Virology, Vaccines and Ser a T orl ak, Belgrade. In contrast to the many other countries, the Serbian vaccine contained four strains: two isolated in 1950s and two in 1980s. Of the four strains, one strain represented prn2 genotype and the other three prn1 genotype. The vaccine has remained unchanged since 1985, when the two newly isolated strains have been added to the vaccine composition. One of the added strains was strain 8/84 with prn2 allele [8]. This unique formulation of the Serbian Pw vaccine provided us an opportunity to study possible effect of the inclusion of “contemporary” strains in the vaccine on temporal trends in B. pertussis population. The finding that frequency of the isolates representing prn2 was low and its appearance was late is striking. Polymorphism in Prn is especially limited to region 1 and is located adjacent to an RDG motif implicated in adhesion [20]. So far, 13 prn alleles have been identified [21]. In many countries the allele prn1 or prn7 is present in most vaccine strains and predominated in pre-vaccine era [14]. However, the “vaccine type” strains were gradually replaced by “non-vaccine type” strains mainly prn2 after the introduction of vaccination. The prn2 is by far the most prevalent type in modern isolates [4,11,12,16,22-24]. Depending on the time when the pertussis vaccination was started and the potency of vaccine used, in most countries isolates containing prn2 were first observed in the 1970s to 1980s [4,9,11,12,23,25]. In line with these observations, in Serbia one isolate (8/84) harboring prn2 was detected in 1984. The isolate was added in vaccine composition in 1985. After that, all isolates were prn1 or prn1 until 2000 when two out of four isolates contained the prn2 allele. The low frequency of prn2 strains and their relatively late emergence in Serbia may be due to the fact that the vaccine contained an isolate having prn2 allele. In this present study, four different prn alleles (prn1, prn2, prn3, and prn11) were detected among the Serbian isolates. The alleles prn1 to prn3 have been observed in many countries [4,12,22,24,26]. The allele prn11 was only reported in a recent study carried out in Australia, where all 5 strains containing prn11 were isolated in the same year (1982) and in the same region [26]. In our study, however, the 6 prn11 isolates were detected in different years from 1984 to 2000. Of the six isolates, four had identical serotype and ptxA genotype. The difference between Prn1 and Prn11 was only one repeat in region 1: Prn1 has five repeats, whereas Prn11 has six repeats. These observations that most of the isolates from 1980 to 2013 contained the prn1 or prn11 allele suggested that the strains prevalent in the pre-vaccine era are still circulating in Serbia. The Serbian vaccine strains do not contain the allele prn3. However, strains harboring the allele prn3 were not isolated in the study at a frequency comparable to that seen in other countries [12,22,24,26]. The exact reasons for the difference are not known. In addition to the vaccine composition, many factors such as immunity, density and dynamics of population can contribute to selection of the circulating strains. So far 8 ptxA alleles have been reported [21]. In most countries, the allele ptxA2 and/or ptxA3 are present in most vaccine strains and predominated in the isolates circulating in pre-vaccine era [4,12,16,23]. However, the “vaccine type” strains were gradually replaced by “non-vaccine type” ptxA1 after the vaccination was introduced. Our result was...
in agreement with the earlier studies. Shift from ptxA2 to ptxA1 was observed in isolates since the late of 1960s, and predominant ptxA genotype in the period 1980-1989 was ptxA1. Interestingly, re-appearance of the ptxA2 allele followed an addition of the two strains harboring ptxA1 in the vaccine composition in 1985. After that moment, there were more ptxA2 isolates than previously. The high frequency of strains harboring ptxA2 in 1990-2013 was not comparable to that noticed in many other countries [4,12,23]. Several studies have shown that Fim2 isolates predominate in unvaccinated population, while they are largely displaced by Fim3 strains when vaccination is introduced with a Pw vaccine containing both Fim2 and Fim3 [27,28]. Although the vaccination has been used in Serbia since 1957, nearly half of the isolates studied from 1957 to 2013 were serotype Fim2. Increases in the incidence of pertussis have been reported in many countries with long vaccination history. Moreover, in many of these countries divergence between vaccine strains and circulating isolates have been found. Interestingly, the incidence of pertussis has been decreasing in Serbia during observed period. It is known that in vaccinated populations, symptoms of pertussis can be mild and the patients do not usually seek for medical help. Therefore, the possibility that incidence of pertussis is underestimated in this country cannot be excluded. Whether the low incidence of pertussis in this country is related to the vaccine used remains to be illustrated. King et al. [29] were the first to show that variation in Prn affects vaccine efficacy in the mouse model [29]. It has been recently shown that the adequate bacterial elimination rates were observed in mice immunized and challenged with the same vaccine type strain [30] and that the vaccine prepared from a recent isolate provided the highest mouse protection when compared to those prepared from the old isolates such as the strain Tohama I [31]. The question of whether prn2 strains eventually became predominant in this country remains to be shown in further investigations. According to the observed findings, B. pertussis population in Serbia during observed period was different from other vaccinated populations and this difference may be related to the vaccine used. The effects of cellular vaccines on the circulating B. pertussis strains should be closely monitored. This study lays a good background for further monitoring of the circulating B. pertussis isolates in Serbia. The exceptionally stable vaccination history with a high vaccination coverage rate makes Serbia a good location for monitoring of the changes in the B. pertussis population after the introduction of a new vaccination program with cellular pertussis vaccines in 2014.

**Figure 6:** Dendrogram analysis of 22 PFGE profiles of B. pertussis isolates circulating in Serbia during 1953–2014. The unweighted pair group method using arithmetic averages (UPGMA) with 1% band tolerance and 1% optimisation settings was used as the clustering method. The symbol $\square$ indicates international reference strains and $\bullet$ Serbian vaccine strains. Of the isolates with identical profiles, only first one is shown.

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References

1. Miller JF, Johnson SA, Black WJ, Beattie DT, Mekalanos JJ, et al. (1992) Constitutive sensory transduction mutations in the Bordetella pertussis bvgS gene. J Bacteriol 174(3): 970-979.
2. Mattos S, Cherry JD (2005) Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infections due to Bordetella pertussis and other Bordetella subspecies. Clin Microbiol Rev 18(2): 326-382.
3. WHO (2012) Immunization, vaccination and biologicals. Vaccine preventable diseases Vaccines monitoring system Global Summary Reference Time Series: SERBIA. World Health Organisation, Switzerland.
4. Cassiday P, Sanden G, Heuvelman K, Mooi FR, Biegard KM, et al. (2000) Polymorphism in Bordetella pertussis pertactin and pertussis toxin virulence factors in the United States, 1935-1999. J Infect Dis 182(5): 1402-1408.
5. Byrne S, Slack AT (2006) Analysis of Bordetella pertussis pertactin and pertussis toxin types from Queensland, Australia, 1999-2003. BMC Infect Dis 6: 53.
6. de Greeff SC, Mooi FR, Westerhof A, Verheuvel JM, Peeters MF, et al. (2010) Pertussis disease burden in the household: how to protect young infants. Clin Infect Dis 50(10): 1339-1345.
7. Celentano EP, Massari M, Paramatti D, Salmosu A, Tozzi AE, et al. (2005) Resurgence of pertussis in Europe. Pediatr Infect Dis J 24(9): 761-765.
8. Dakic G, Kallonen T, Elomaa A, Pjessa T, Krastavcevic VM, et al. (2010) Bordetella pertussis vaccine strains and circulating isolates in Serbia. Vaccine 28(5): 1180-1192.
9. Mooi FR, Hallander H, Konig WCH, Hoet B, Guiso N (2000) Epidemiological typing of Bordetella pertussis isolates: recommendations for a standard methodology. Eur J Clin Microbiol Infect Dis 19(3): 174-181.
10. Bouchez V, Caro V, Levillain E, Guigon G, Guiso N (2008) Genomic content of Bordetella pertussis clinical isolates in areas of intensive children vaccination. PLoS One 3(6): e2437.
11. Advani A, Donnelly D, Hallander H (2004) Reference system for characterization of Bordetella pertussis pulsed-field gel electrophoresis profiles. J Clin Microbiol 42(7): 2890-2897.
12. Elomaa A, Advani A, Donnelly D, Antila M, Mertsola J, et al. (2005) Strain variation among Bordetella pertussis isolates in Finland, where the whole-cell pertussis vaccine has been used for 50 years. J Clin Microbiol 43(8): 3681-3687.
13. Heikkinen E, Xing DK, Olander RM, Hytönen J, Viljanen MK, et al. (2008) Bordetella pertussis isolates in Finland: serotype and fimbral expression. BMC Microbiol 8:162.
14. He Q, Mertsola J (2008) Factors contributing to pertussis resurgence. Future Microbiol 3(3): 329-339.
15. Mooi FR, He Q, van Oirschot K, Mertsola J (1999) Variation in the Bordetella pertussis virulence factors pertxin and pertactin in vaccine strains and clinical isolates in Finland. Infect Immun 67(6): 3133-3134.
16. Kallonen T, Hamnukela GYK, Elomaa A, Lubytska A, Fry NK, et al. (2011) Differences in the genomic content of Bordetella pertussis isolates before and after introduction of pertussis vaccine in four European countries. Infect Gen and Evol 11(8): 2034-2042.
17. Halperin SA (2007) The control of pertussis-2007 and beyond. N Engl J Med 356(2): 110-113.
18. McIntyre P, Gidding H, Gilmour R, Lawrence G, Hull B, et al. (2002) Vaccine preventable diseases and vaccination coverage in Australia, 1999 to 2000. Commun Dis Intell Suppl-Suppl: 1-11.
19. Mooi FR, van Loo IH, King AJ (2001) Adaptation of Bordetella pertussis to vaccination: a cause for its reemergence? Emerg Infect Dis 7(3): 526-528.
20. Leininger E, Roberts M, Kenimer JG, Charles IG, Fairweather N, et al. (1991) Pertxin, an Arg-Gly-Asp-containing Bordetella pertussis surface protein that promotes adherence of mammalian cells. Proc Natl Acad Sci USA 88(2): 345-349.
21. Mooi FR (2010) Bordetella pertussis and vaccination: The persistence of a genetically monomorphic pathogen. Infect Genet Evol 10(1): 36-49.
22. Mooi FR, van Oirschot K, Heuvelman K, van der Heide HG, Gaastra W, et al. (1998) Polymorphism in the Bordetella pertussis virulence factors P69/pertactin and pertussis toxin in The Netherlands: temporal trends and evidence for vaccine driven evolution. Infect Immun 66(2): 670-675.
23. Weber C, Eade BC, Coralie G, Caro V, Guiso N (2001) Polymorphism of Bordetella pertussis isolates circulating for the last 10 years in France, where a single effective whole-cell vaccine has been used for more than 30 years. J Clin Microbiol 39(12): 4396-4403.
24. Njamkepo E, Cantinelli T, Guigon G, Guiso N (2008) Genomic analysis and comparison of Bordetella pertussis isolates circulating in low and high vaccine coverage areas. Microbes Infect 10(14-15): 1582-1586.
25. Hallander HO, Advani A, Donnelly D, Gustafsson L, Carlsson RM (2005) Shifts of Bordetella pertussis variants in Sweden from 1970 to 2003, during three periods marked by different vaccination programs. J Clin Microbiol 43(6): 2856-2865.
26. Poynten M, McIntyre PB, Mooi FR, Heuvelman KJ, Gilbert GL (2004) Temporal trends in circulating Bordetella pertussis strains in Australia. Epidemiol Infect 132(2): 185-193.
27. Hallander H, Advani A, Riffelmann M, von König CH, Caro V, et al. (2007) Bordetella pertussis strains circulating in Europe in 1999 to 2004 as determined by pulsed-field gel electrophoresis. J Clin Microbiol 45(10): 257-262.
28. Preston NW, Carter EJ (1992) Serotype specificity of vaccine-induced immunity to pertussis. Communicable disease report 2(13): R155-156.
29. KingAJ, Berbers G, van Oirschot HF, Hoogerhout P, Knipping K, et al. (2001) Role of the polymorphic region 1 of the Bordetella pertussis protein pertactin in immunity. Microbiology147(11): 2885-2895.
30. Bottero D, Gaillard ME, Fingermann M, Welman G, Fernández J, et al. (2007) Pulsed-field gel electrophoresis, pertactin, pertussis toxin SI subunit polymorphisms, and surfaceome analysis of vaccine and clinical Bordetella pertussis strains. Clin Vaccine Immunol 14(11): 1490-1498.
31. Pereira A, Pereira AS, Filho MCA, Bando SY, Tambourgi DV (2005) Comparative analysis of a Bordetella pertussis patient isolated strain and classical strains used in the pertussis vaccine. Vaccine 23(34): 4353-4358.

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