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CHANGES IN GENETIC DIVERSITY OF THE *BORDETELLA PERTUSSIS* POPULATION IN SERBIA BETWEEN 1953 AND 2011

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Abstract - 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. To monitor changes in bacterial population, 77 isolates collected from 1953 to 2011 were studied. The methods included serotyping of fimbriae (Fim), genotyping of pertactin (prn) and pertussis toxin S1 subunit (ptxA). A shift from ptxA2 to ptxA1 has been observed in isolates since the late of 1960s. In the period 1961-1979, the genotype ptxA1 became as common as genotype ptxA2. After that, during the period 1980-1989, the predominant ptx genotype was ptxA1. The reappearance 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 in three isolated strains from 2011. Serotype Fim2.3 disappeared before 1980 and serotype Fim2 became predominant thereafter. The results of this 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.

Key words: *Bordetella pertussis*, pertussis toxin, pertactin

INTRODUCTION

Pertussis (whooping cough) is a worldwide infectious disease caused by the bacteria *Bordetella pertussis* (Miller et al., 1992). Despite the fact that the introduction of vaccination in the 1950s and 1960s has reduced pertussis morbidity and mortality, this disease is still prevalent (Mattoo and Cherry, 2005) and pertussis is still one of the leading causes of vaccine-preventable deaths in the world (WHO, 2012). 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 (Cassiday et al., 2000; Mattoo and Cherry, 2005; Byrne and Slack, 2006; de Greeff et al., 2010, Celentano et al., 2005). 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 implemented for 50 years. The diphtheria-tetanus-whole cell pertussis (DTPw) vaccine has been manufactured in the Institute of Virology, Vaccine and Sera Torlak, Belgrade, Serbia since 1957. The DTPw vaccine is given at 2, 4, 6, and 12 months of age. A second booster dose with the mono pertussis vaccine was given at the age of 4 years during the periods from 1970 to 1981 and from 1990 to 2000. The current composition of the vaccine has been used since 1985 and contains four *B. pertussis* strains. The vaccine strains were chosen in compliance with serotype, immunogenicity and specific toxicity. The four strains represent three serotypes: Fim2 (8/84), Fim2.3 (1772/57 and 2047/57) and Fim3 (23/81). The vaccine strains 2047/57 and 1772/57 represent ptxA2/prn1 genotypes, whereas the vaccine strains 23/81 and 8/84 harbor ptxA1/prn1 and ptxA1/prn2. All vaccine strains are in equal amount in the vaccine composition (Dakic et al., 2010). The reported vaccination coverage in Serbia ranged from 79% to 98% (median, 90%) in 1981-2011 (WHO, 2012). The aim of this study was to analyze *B. pertussis* isolates circulating between 1953 and 2011 in Serbia by standard typing methods (Mooi et al., 2000) and to compare them to 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 strain and clinical isolates is 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 for 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 72 h on Bordet Gengou agar supplemented with 30% defibrinated sheep blood and subcultured on the same medium for 24 h (Bouchez et al., 2008).

Fimbriae serotyping of B. pertussis isolates

Serotyping was performed with monoclonal antibodies against Fim2 and Fim3 by slide agglutination test (Advani et al., 2004; Mooi et al., 2000)

Genotyping of Ptx S1 subunit (ptxA) and prn

The standardized genotyping of Ptx S1 subunit (ptxA) and prn was performed by sequencing and LightCycler PCR (Advani et al., 2004; Elomaa et al., 2005; Heikkinen et al., 2008). These methods have been recommended for the epidemiological typing of *B. pertussis* isolates (Mooi et al., 2000).

Statistical analysis

The chi-square test was used to compare frequencies of strain genotypes and serotypes between four time-periods (1953-1960, 1961-1979, 1980-1989 and 1990-2011). A p-value ≤ 0.05 was considered 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 (Fig. 1). All isolates prior to 1960 were recovered from unvaccinated patients. In the period 1980-1989, 82.35% of patients were vaccinated while 64.3% were vaccinated in the period 1990-2011. 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 (Supplementary Data). The frequency of each serotype has changed over time. Before the introduction of vaccination,

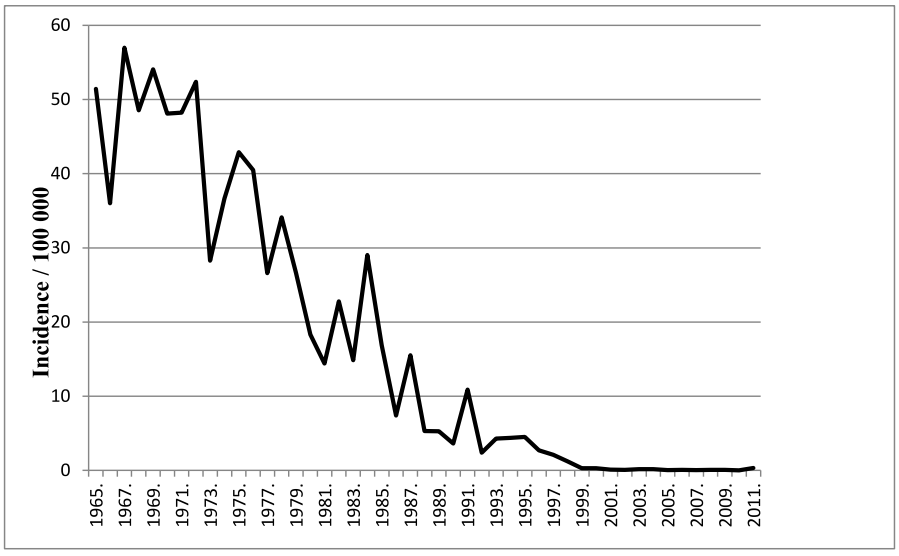

Fig. 1. The incidence (cases per 100 000) of reported pertussis cases in Serbia between 1965 and 2011. Source: Infectious Disease Register, Institute of Public Health of Serbia "Dr Milan Jovanovic Batut".

the prevalent serotypes were Fim2 (38%) and Fim2.3 (62%). After the introduction of vaccination, the frequency of serotype Fim2.3 decreased, being significantly lower (0% in 1980-1989 and 23% in 1990-2011) 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.

Genotypes of ptxA and prn

All strains isolated from 1953 to 1960 were of ptxA2 genotype. A shift from ptxA2 to ptxA1 has been observed in isolates since the late 1960s. In the period 1961-1979, the genotype ptxA1 became as common as genotype ptxA2. After that, during the period 1980-1989, the predominant ptx genotype was ptxA1 (91.2%). The re-appearance of isolates containing ptxA2 was noticed after the two strains harboring ptxA1 were added into the vaccine in 1985 (Dakic et al., 2010). During the period of 1990-2011, both ptxA genotypes were present in the population.

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

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

DISCUSSION

Vaccinations with Pw vaccines were introduced

SUPPLEMENTARY DATA

Strain code	Gender	Age (years)	**Vaccination Status**	**Serotype**	**Prn**	**PtxA**	**Year of isolation**	**Note**
22/53	f	5	no	2	1	2	1953	
124/54	N.A.	N.A.	no	2	1	2	1954	
162/54	m	8	no	2,3	1	2	1954	
177/54	m	7	no	2,3	1	2	1954	
200/54	f	4	no	2,3	1	2	1954	
38/54	f	7	no	2	1	2	1954	
40/54	m	5	no	2	1	2	1954	
515/55	N.A.	N.A.	N.A.	2	1	2	1955	
1078/56	f	2.5	no	2,3	1	2	1956	
1499/56	N.A.	N.A.	N.A.	2,3	1	2	1956	
1496/57	N.A.	N.A.	no	2	1	2	1957	
1611/57	f	3	no	2	1	2	1957	
1714/57	m	4.5	no	2,3	1	2	1957	
1772/57	N.A.	N.A.	N.A.	2,3	1	2	1957	vaccine strain since 1972
1828/57	f	4 months	no	2,3	1	2	1957	
1881/57	N.A.	N.A.	no	2,3	1	2	1957	
2047/57	N.A.	N.A.	N.A.	2,3	1	2	1957	vaccine strain since 1968
2048/57	m	6	no	2	1	2	1957	
1997/58	m	4.5	no	2,3	1	2	1958	
2154/58	f	7	no	2,3	1	2	1958	
3008/60	N.A.	N.A.	N.A.	2,3	1	2	1960	
33/69	N.A.	N.A.	N.A.	3	1	1	1969	
90/69	N.A.	N.A.	N.A.	2,3	1	2	1969	
108/69	N.A.	N.A.	N.A.	2,3	1	2	1969	

SUPPLEMENTARY DATA *continued*

Strain code	Gender	Age (years)	**Vaccination Status**	**Serotype**	**Prn**	**PtxA**	**Year of isolation**	**Note**
122/69	N.A.	N.A.	N.A.	2,3	1	2	1969	
258/70	N.A.	N.A.	N.A.	2,3	1	2	1970	
487/71	N.A.	N.A.	N.A.	2,3	1	2	1971	
375/71	N.A.	N.A.	N.A.	2,3	1	1	1971	
849/73	N.A.	N.A.	N.A.	3	1	1	1973	
885/73	N.A.	N.A.	N.A.	3	1	1	1973	
55145/80	N.A.	N.A.	N.A.	3	1	1	1980	
59377/80	N.A.	N.A.	N.A.	3	1	1	1980	
23/81	f	3	yes, no booster	3	1	1	1981	vaccine strain since 1985
29/81	m	6	yes	3	1	1	1981	
30/81	f	3	yes	3	3	1	1981	
32/81	m	9 months	1 dose	3	1	1	1981	
40311/81	f	7	yes	3	1	1	1981	
1/84	m	6	yes, no booster	2	1	1	1984	
2/84	f	6	yes	2	1	1	1984	
3/84	f	4	yes	2	1	1	1984	
4/84	f	6	yes	2	1	1	1984	
5/84	m	6	yes	2	3	1	1984	
6/84	m	1	yes	2	1	1	1984	
7/84	f	5	yes	2	1	1	1984	
8/84	m	4 months	no	2	2	1	1984	vaccine strain since 1985
9/84	f	33	yes	2	1	1	1984	
10/84	m	N.A.	no	2	1	1	1984	
11/84	m	1	no	3	1	1	1984	
12/84	m	1	yes	2	1	1	1984	

SUPPLEMENTARY DATA *continued*

Strain code	Gender	Age (years)	**Vaccination Status**	**Serotype**	**Prn**	**PtxA**	**Year of isolation**	**Note**
14/84	m	13	yes	2	1	1	1984	
15/84	m	2	yes	2	1	1	1984	
17/84	m	12	yes	2	1	1	1984	
18/84	m	16 months	2 doses	2	1	1	1984	
20/84	f	5	yes	3	11	1	1984	
21/84	f	10	yes	2	1	1	1984	
22/84	m	9	yes	3	1	1	1984	
24/84	f	4	yes	3	1	1	1984	
28/85	m	6	yes	2	1	1	1985	
50042/86	m	2	no	2	11	2	1986	
52121/86	m	3 months	no	2	1	1	1986	
9783/87	f	4.5	yes	2	1	2	1987	
28270/89	f	10	yes	2	11	2	1989	
28756/89	f	9	yes	3	3	1	1989	
28928/89	f	6	yes	2	1	1	1989	
12/90	m	11	yes	2	11	2	1990	
51651/90	m	10	yes	2	11	2	1990	
52376/90	f	11	yes	2	1	1	1990	
53367/90	f	9	yes	2	1	1	1990	
9420/90	m	12	yes	2	1	1	1990	
53746/91	m	6 months	1 dose	2	1	1	1991	
1/00	m	13	yes	2	11	2	2000	
6/00	m	1.5	2 doses	3	3	1	2000	
7/00	m	9	yes	3	2	1	2000	

SUPPLEMENTARY DATA *continued*

Strain code	Gender	Age (years)	**Vaccination Status**	**Serotype**	**Prn**	**PtxA**	**Year of isolation**	**Note**
8/00	m	12	yes	3	2	1	2000	
2/11	m	2.5 months	no	2,3	2	1	2011	
3/11	f	2,5	no	2,3	2	1	2011	
5/11	m	2 months	no	2,3	2	1	2011	

Abbreviations:
N.A.: not available
m: male
f: female

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*	*prn 11*	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
1990-2011	13	10	3	4	5	1	3	7	3	3
Total	77	45	32	61	6	4	6	37	22	18

from the 1940s to the 1960s. They have successfully reduced morbidity and mortality from pertussis throughout the world (He et al., 2008). Pw vaccines have been produced by different manufacturers, and the *B. pertussis* vaccine strains vary. The vaccine strains, both for whole-cell and acellular vaccines, were isolated from the 1940s to the 1960s, and in many countries the vaccinations have selected circulating isolates that were dissimilar to the vaccine strains (Elomaa et al., 2005; Cassiday et al., 2000; Mooi et al., 1999, Kallonen et al., 2011). Furthermore, the resurgence of pertussis has been observed in countries with long-term pertussis vaccination (Cellentano et al., 2005; Halperin et al., 2007; McIntyre et al., 2002; Mooi et al., 2001, Kallonen et al., 2011). In Serbia, the Pw vaccine has been in use from 1957 and is manufactured in the Institute of Virology, Vaccines and Sera Torlak, Belgrade. In contrast to the many other countries, the Serbian vaccine contains four strains: two isolated in the 1950s and two in the 1980s. Of the four strains, one strain carries the prn2 genotype and the other three the prn1 genotype. The vaccine has remained unchanged since 1985, when two newly isolated strains were added to the vaccine composition. One of the added strains was strain 8/84 with the prn2 allele (Dakic et al., 2010). This unique formulation of the Serbian Pw vaccine provided us an opportunity to study the possible effect of the inclusion of "contemporary" strains in the vaccine on temporal trends in the *B. pertussis* population. The discovery that the frequency of Serbian isolates representing

prn2 was low and its appearance was late, is striking. Polymorphism in Prn is essentially limited to region 1 and is located adjacent to an RDG motif implicated in adhesion (Leininger et al., 1991). So far, 13 prn alleles have been identified (Mooi, 2010). In many countries, the allele prn1 or prn7 is present in most vaccine strains and predominated in the pre-vaccine era (He et al., 2008). However, the "vaccine type" strains were gradually replaced by "non-vaccine type" strains, mainly prn2, after the introduction of vaccination. Prn2 is by far the most prevalent type in modern isolates (Advani et al., 2004; Elomaa et al., 2005; Cassiday et al., 2000; Mooi et al., 1998; Weber et al., 2001; Njamkepo et al., 2008; Kallonen et al., 2011). Depending on the time when pertussis vaccination was started and the potency of the vaccine used, in most countries isolates containing prn2 were first observed from the 1970s to the 1980s (Advani et al., 2004; Elomaa et al., 2005; Cassiday et al., 2000; Mooi et al., 1998; Weber et al., 2001; Hallander et al., 2005). In line with these observations, in Serbia one isolate (8/84) harboring prn2 was detected in 1984. The isolate was added to the vaccine composition in 1985. After that, all isolates were prn1 or prn11 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 because the vaccine contains an isolate with a prn2 allele. In this present study, four different prn alleles (prn1, prn2, prn3 and prn11) were detected among the Serbian isolates. The alleles prn1-3 have been observed in many countries (Elomaa et al., 2005; Cassiday et al., 2000; Mooi et al., 1998; Weber et al., 2001; Poynten et al., 2004; Njamkepo et al., 2008). The allele prn11 was only reported in a recent study carried out in Australia, where all five strains containing prn11 were isolated in the same year (1982) and the same region (Poynten et al., 2004). However, in our study, the six prn11 isolates were detected in different years from 1984 to 2000. Of the six isolates, four had identical serotypes and the ptxA genotype. The difference between Prn1 and Prn11 was only one repeat in region 1. Prn1 has five repeats, whereas Prn11 has six repeats. The observation that most of the isolates from 1980 to 2011 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 prn3 allele were not isolated in the study at a frequency comparable to that seen in other countries (Elomaa et al., 2005; Mooi et al., 1998; Poynten et al., 2004; Njamkepo et al., 2008) 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, eight ptxA alleles have been reported (Mooi, 2010). 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 (Elomaa et al., 2005; Cassiday et al., 2000; Weber et al., 2001; Kallonen et al., 2011). However, the "vaccine type" strains were gradually replaced by "non-vaccine" type ptxA1 after vaccination was introduced. Our result is in agreement with earlier studies. A shift from ptxA2 to ptxA1 was observed in isolates since the late 1960s, and the predominant ptxA genotype in the period 1980-1989 was ptxA1. Interestingly, the reappearance of the ptxA2 allele followed an addition of the two strains harboring ptxA1 to the vaccine composition in 1985. After that moment, there were more ptxA2 isolates than previously. The high frequency of strains harboring ptxA2 in 1990-2011 was not comparable to that noticed in many other countries (Elomaa et al., 2005; Cassiday et al., 2000; Weber et al., 2001). Several studies have shown that Fim2 isolates predominate in unvaccinated populations, while they are largely displaced by Fim3 strains when vaccination is introduced with a Pw vaccine containing both Fim2 and Fim3 (Hallander et al., 2007; Preston et al., 1992). Although vaccination has been implemented in Serbia since 1957, nearly half of the isolates studied from 1957 to 2011 were serotype Fim2. Increases in the incidence of pertussis have been reported in many countries with a long vaccination history. Moreover, in many of these countries divergence between vaccine strains and circulating isolates has been detected. Interestingly, the incidence of pertussis has been decreasing in Serbia. It is known that in vaccinated populations the symptoms of pertussis

can be mild and patients do not usually seek medical help. Therefore, the possibility that the 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 confirmed. King et al. (2001) were the first to show that variation in Prn affects vaccine efficacy in the mouse model. It has been recently shown that the adequate bacterial elimination rates were observed in mice immunized and challenged with the same vaccine type strain (Bottero et al., 2007), 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 (Pereira et al., 2005). The question of whether prn2 strains eventually became predominant in this country remains to be uncovered in further investigations. According to the observed findings, the *B. pertussis* population in Serbia is different from other vaccinated populations, and this difference may be related to the vaccine used.

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