



Pertussis characterization in the central-west region of São Paulo state, Brazil

Caracterização da coqueluche na região centro-oeste do Estado de São Paulo, Brasil

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ABSTRACT

Pertussis is a highly contagious respiratory disease caused by *Bordetella pertussis*. This study aimed at characterizing the *B. pertussis* laboratory positivity and the isolated strains in municipalities of the Central-West Region of São Paulo State, Brazil from 2010 to 2014. A total of 597 nasopharyngeal swabs samples were collected from suspected patients and contacts, and analyzed by *in vitro* culture and Real-Time PCR (qPCR). Culture-positive *B. pertussis* strains were characterized by serotyping and pulsed-field gel electrophoresis. Considering culture and/or qPCR, the positivity rate was of 19.6%. Out of 117 samples with *B. pertussis*, 23 were detected by both methods, 89 by qPCR only and five by culture only. Strains presenting FIM3 (40%), FIM2,3 (32%) and FIM2 (28%) serotypes were found. Five pulsotypes were detected by PFGE, 48% of which identified as BP.Xba.0039, being the predominant type in this study. Among the positive strains, 50% were isolated from <2 months old-children and 17% were isolated from three to six months old patients. Non-vaccinated children or with incomplete vaccination schedule were at the major risk of complications and death, highlighting the importance of a continuous monitoring of this infection for the future control strategies.

Keywords. *Bordetella pertussis*, whooping cough, pulsed field gel electrophoresis, real-time PCR.

RESUMO

A coqueluche é uma doença respiratória altamente contagiosa causada por *Bordetella pertussis*. Este estudo caracterizou a positividade de *B. pertussis* e as cepas isoladas em municípios da Região Centro-Oeste do Estado de São Paulo de 2010 a 2014. Foram coletados 597 esfregaços nasofaríngeos de pacientes e contatos suspeitos de coqueluche, e analisados por cultura e Real-Time PCR (qPCR). Os isolados de *B. pertussis* obtidos de cultura foram caracterizados por sorotipagem e eletroforese em gel de campo pulsado. Considerando-se a cultura e/ou qPCR, verificou-se taxa de positividade de 19,6%. Das 117 amostras positivas para *B. pertussis*, 23 foram detectadas por ambos os métodos, 89 apenas por qPCR e cinco apenas na cultura. Foram detectadas cepas de sorotipos FIM3 (40%), FIM2,3 (32%) e FIM2 (28%). Cinco pulsotipos foram detectados pela PFGE, e 48% identificados como BP.Xba.0039, o tipo predominante neste estudo. Entre as cepas positivas, 50% foram isoladas de crianças menores de dois meses e 17% isoladas da faixa etária de três a seis meses. Crianças não vacinadas ou com esquema de vacinação incompleta têm maior risco de complicações e óbito, o que ressalta a importância do monitoramento contínuo desta infecção para futuras estratégias de controle.

Palavras-chave. *Bordetella pertussis*; coqueluche, eletroforese em gel de campo pulsado, PCR em tempo real.

INTRODUCTION

Whooping cough or pertussis is an acute infectious disease which harms the human upper respiratory tract caused by *Bordetella pertussis*^{1,2}. There are ten genetically distinct species in the genus *Bordetella*, including *B. pertussis*, *B. parapertussis*, *B. holmesii* and *B. bronchiseptica* as the most commonly associated with human respiratory infections³. *B. bronchiseptica* rarely infects the healthy individuals and *B. holmesii* is likely to cause disease mainly in adults^{4,5}, although there are reports on this bacterium circulating among infant populations⁶.

Vaccination constitutes the main form for controlling and preventing pertussis. The heat-inactivated *B. pertussis* organisms and the acellular vaccine are composed of highly purified selected components⁷.

Despite the high vaccination coverage, *B. pertussis* continues to circulate in both developed and developing countries, and causing outbreaks in every age groups, with epidemic cycles every 3-5 years^{8,9}. In Brazil the reemergence of pertussis occurred from 2011 to 2014 and high number of cases were reported, including outbreaks descriptions in Distrito Federal in 2012 and 2014¹⁰. In 2015, 10,487 suspected cases were reported and 28.2 % were confirmed. The incidence rate was 1.5 cases per 100,000 inhabitants. The Brazilian states with the highest number of reported cases in that year were São Paulo (4,170), Paraná (883) and Pernambuco (710)¹¹.

Many hypotheses have been suggested to explain the reemergence of pertussis in various countries, including Brazil, such as, the use of better laboratory diagnostic tools, the improvement in epidemiological surveillance, the reduction in the vaccine efficacy and the pathogen adaptation or genetic changes due to the selective pressure caused by acellular vaccines^{12,13}. Furthermore, the reemergence of the disease could be the consequence of progressive loss of immunity, rendering the individuals susceptible over the years. These groups are prone to act as bacteria reservoirs and as infection sources for lactating children younger than six months old, who are still incompletely vaccinated^{14,15,16}.

The clinical pertussis case definitions require the occurrence of respiratory symptoms, such as paroxysmal cough for at least two weeks, the inspiratory whoop and the post-tussive emesis. However, the specificity of case definitions is negatively influenced by the time from infection to diagnosis, by the previous vaccination or infection, and by increasing the age of patients¹⁷. Clinical symptoms are not pathognomic, therefore the laboratory diagnosis is recommended whenever is possible¹⁸.

The laboratory diagnosis of pertussis can be confirmed by microbiological, immunological and molecular methods, as *in vitro* culture of nasopharyngeal secretions, detection of anti-*Bordetella* antibodies and Real-Time PCR (qPCR), respectively^{2,19}. The use of molecular techniques has improved the sensitivity of the pertussis laboratorial detection. However, the culture has still been considered as the gold standard method for diagnosing, and it should be maintained in the laboratory routine²⁰.

Studies using the molecular typing techniques as Pulsed-field Gel Electrophoresis (PFGE), Multilocus Sequence Typing (MLST) and Multilocus Variable Number Tandem Repeat Analysis (MLVA) show the changes occurred in the *B. pertussis* circulating in the world. The continuous monitoring of these strains is needed for controlling and preventing the strategies of the disease^{21,22}.

The present study aimed at evaluating the laboratory pertussis positivity in the Central-West municipalities of São Paulo State, Brazil during the period from 2010 to 2014, characterizing the cultured strains by serotyping and PFGE techniques.

MATERIAL AND METHODS

Samples

From January 2010 to December 2014, 597 nasopharyngeal swabs samples were analyzed in the Regional Laboratory of Marília (CLR/IAL – Marília, SP). These samples were collected from the pertussis-suspected patients and their contacts (age range being from four days to 65 years old), in accordance with the definition of suspected case recommended by the Ministry of Health of Brazil^{23,24}.

The samples came from hospitals (n = 441) and outpatient health units (n = 156). Two hundred and fifty-seven samples were collected from patients, seven from communicants and in 333 no information was available. These patients were treated in 47 hospitals settled in 15 municipalities in the region of Marília city, located in the center-west area of São Paulo State. Patients presenting incomplete data (no report on the date of symptom onset and/or no registration on the dates of sample collection) were excluded from this study. The analyzed variables were age, sex and calendar year of isolation.

Isolation and Identification of *B. pertussis*

Samples were collected by using the flexible, sterile alginate swabs and then transported to the CLR/IAL-Marília in semi-solid charcoal agar (Regan-Lowe - RL) supplemented with 10 % sheep blood and 40 µg/mL cephalexin²⁵. The nasopharyngeal swabs samples were cultured in the same collection day on RL agar; and they were incubated at 35 °C- 37 °C up to ten days, under an air ambient with high humidity. Colonies suspected of belonging to the genus *Bordetella* were confirmed by Gram staining, and the species were identified by biochemical tests previously described²⁶. Serotyping was used to detect the O-antigen and the fimbrial antigens FIM2 and FIM3 from the *B. pertussis* strains by slide agglutination, following Gonçalves et al²⁶. Anti-serum O1 and fimbrial antibodies FIM2 and FIM3 were produced in the National Reference Laboratory for Pertussis - Instituto Adolfo Lutz (IAL), São Paulo-SP, Brazil.

Real Time PCR (qPCR)

After culturing, the swabs were placed into dry, sterile tubes and stored at -20 °C until they were shipped to IAL- São Paulo for performing the DNA and qPCR. DNA extraction was carried out by following the manufacturer recommendations using a MagNA Pure LC instrument with MagNA Pure LC DNA Isolation Kit I (Roche Applied Science, Indianapolis, IN).

The qPCR methodology used in the present study was proposed by Leite et al²⁷. The reaction was carried out in a LightCycler[®]480 Software release 1.5.0 SP3 - Roche[®] thermal cyler, including the primers and the probes specific for detecting the *ptxS1* toxin

gene (GenBank n° AJ920066) and the IS481 insertion element (GenBank n° M22031), present in various copies in the *B. pertussis* chromosome.

Pulsed Field Gel Electrophoresis (PFGE)

The strains of *B. pertussis* were analyzed by means of the PFGE technique proposed by Advaniet al²⁸ using the restriction enzyme *Xba*I, with few modifications as follows. PFGE was carried out at 6V/cm, 14 °C, in two blocks, with a 5-second initial pulse and a 6-second final pulse for 11 hours (block 1), and 8-second initial pulse and 35-second final pulse for 13 hours (block 2). The band pattern was analyzed using the BioNumerics software package (ver. 7.1; Applied Maths, Inc.), and the similarity index was determined by the Dice similarity coefficient and Unweighted Pair Group Method using Arithmetic averages grouping.

Ethical Aspects

This study followed the recommendations provided by Resolution no. 466 of 2012 – National Health Council for Clinical Research in Humans; and it was approved by the Ethics Committee and being registered at the *Plataforma Brasil* under ID number CAAE: 49836815.0.0000.0059.

RESULTS

During the period from 2010 to 2014 a significant increase in the number of laboratorial analyses was noticed (5, 43, 74, 152, and 323 samples in 2010, 2011, 2012, 2013 and 2014, respectively), indicating an expansion in the laboratory surveillance of pertussis in the region. However, in spite of improving the surveillance and analyzing higher number of samples, the positivity rates have remained consistent (approximately 20 %) since the introduction of the qPCR methodology in 2010 (**Figure 1**).

Of 597 analyzed samples, 117 (19.6 %) were positive for *B. pertussis* by culture and/or qPCR; qPCR detected 89 (76 %) samples, culture 5 (4 %) and qPCR and culture simultaneously detected 23 (20 %) positive samples. Among the culture-positive samples, 25 were serotyped and characterized by PFGE (three strains were unviable after performing several attempts of recovery).

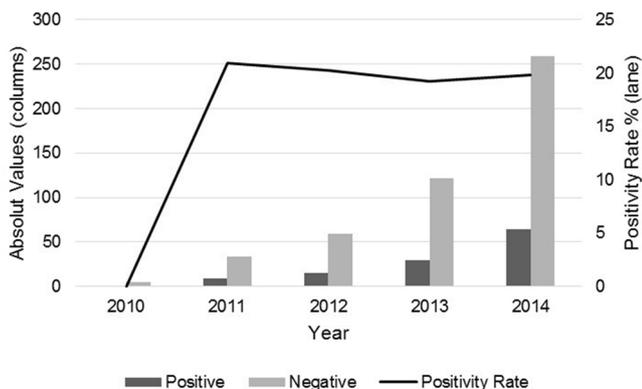


Figure 1. Sample number and positivity rate of *Bordetella pertussis* in nasopharyngeal swabs of suspected patients from the Central-West Region of São Paulo State, from 2010 to 2014

The age group presenting the highest number of positive results and also with the highest number of collected samples was that from children under two months (56/159, 35 %) followed by the group of children aging from three to six months (34/121, 28 %). Regarding to gender, 58 % were collected from female and 42 % from male with positivity rate of 10 % and 9 %, respectively.

According to the serotype, this study showed a predominance of serotype FIM3 strains (10 strains; 40 %) followed by serotype FIM2,3 (8 strains; 32 %) and FIM2 (7 strains; 28 %).

Analysis by PFGE showed five different restriction profiles (pulsotypes) which were identified by the restriction of genomic DNA by the enzyme XbaI (Figure 2), and a similarity of 88.3 % was found among the isolates. The most frequent profile was BP.Xba.0039 (12/25 isolates), distributed in the years 2012, 2013 and 2014.

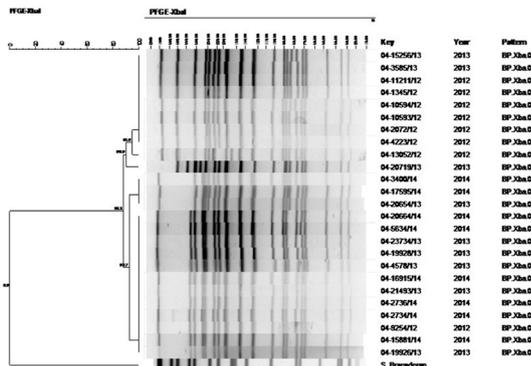


Figure 2. Restriction patterns and dendrogram of *Bordetella pertussis* isolates from the Central-West Region of São Paulo State, from 2012 to 2014

The BP.Xba.0028 profile (8/25 isolates) was also prevalent, but it was isolated in 2012 and 2013 only. The other profiles were BP.Xba.0026 (n = 1 in 2013), BP.Xba.0027 (n = 1 in 2012) and BP.Xba.0040 (n = 3, in 2013 and 2014) (Figure 3).

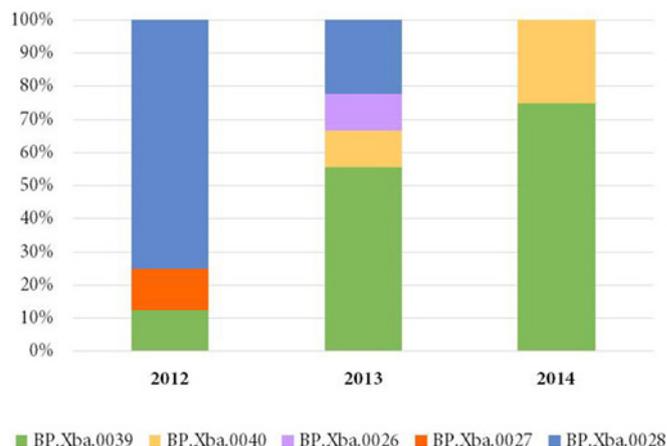


Figure 3. Temporal distribution of the *Bordetella pertussis* pulsotypes from the Central-West Region of São Paulo State, from 2012 to 2014, determined by pulsed-field gel electrophoresis

DISCUSSION

Whooping cough is one of the most common diseases affecting neonates, children and adults, and in the recent years an increase in the cases number has been detected. And this disease has been a meaningful cause of morbidity and mortality worldwide³. Among the vaccine-preventable diseases, pertussis is the fifth leading cause of death in children under five years old, and according to WHO estimates in 2013, approximately 63,000 deaths occurred among patients of this age group⁸.

In the present study, the qPCR showed higher positivity rate when compared to *B. pertussis* culture. Among the laboratory diagnosis techniques, the molecular assays show highest sensitivity and rapid diagnosis, which positively impact the surveillance of this disease. And they contribute to the higher agility to the prevention actions. Although the pertussis diagnosis might be performed following the clinical and the clinical-epidemiological criteria, according to Zouari et al¹⁸, the pertussis clinical signs are not pathognomonic, which enhances the laboratory diagnostic significance.

This study showed higher positivity in the pertussis laboratory diagnosis in children under two months of age and in children under six months of age, who were not vaccinated or received an incomplete vaccination schedule. These circumstances put them mostly to the risk of complications and death, corroborating the previous studies which showed the correlation between the high rates of positivity and the death in these groups^{9,27,29,30}.

Several studies have shown the resurgence of pertussis in many countries in the last 10 years^{22,31}. One of the hypotheses for this reemergence would be the substitution of the whole cell vaccine by the acellular vaccine in many developed countries. This vaccine presents a restrict immunogenic protein variety, which could have caused a selection within the circulating clones of *B. pertussis*; and in consequence the patient numbers rise up²². In Brazil, the whole-cell vaccine has still being used for immunizing against this disease³². Thus, it could explain the results found in the present study, in which a proportional increase in the pertussis laboratory positivity has not observed over the years in the analyzed region, but the positivity rate is remaining stable. On the other hand, in other Brazilian locations, an increase in the number of cases was observed, which could be correlated with other factors beyond the vaccine, according to Torres et al²⁹.

The results detected in this study by serotyping showed the predominance of serotype Fim3 corroborating the data from the previous studies. Thus, it is suggested that this serotype has been prevalent in Brazil and in other countries for decades^{14,22,26,27,33}.

According to the temporal distribution of the pulsotypes, it was observed the replacement of the pulsotype BP.Xba.0028, predominant in 2012, by the BP.Xba.0039 and BP.Xba.0040 types. This study showed the predominance of the pattern BP.Xba.0039, which gradually increased from 2012 (12.5 %, 1/8 strains) to 2013 (55.6 %, 5/9) and 2014 (75 %; 6/8). It is interesting to note the homogeneity of two pulsotypes only in 2014, BP.Xba.0039 and BP.Xba.0040, presenting a similarity value of 92.7 %.

Similar data were reported in the USA³⁴, and the predominance of one clone circulating from 2000 to 2012 was described. In England, a decrease in genetic diversity along with the predominance of few clones was observed, which started with the introduction of acellular vaccine³⁵. The predominance of one clone could be associated with the antigenic selection promoted by vaccination or mutations, which increase the virulence and persistence of such clone in patients and infected individuals³⁶.

CONCLUSION

The present study has shown that the rates of laboratory positivity for pertussis have remained constant in the last years in the Central-West Region of São Paulo State. The predominance of the same serotype is reported, however with the substitution of the circulating clones during the years of the study. Children aged less than two months of age and from three to six months were the most affected group, because they were not vaccinated yet or they received incomplete immunization schedule. Strategies for preventing and controlling the disease as the suspected cases notification, the continued laboratory monitoring and the prevention measures as maternal vaccination recommended by the Brazilian Ministry of Health are fundamental tools for protecting the newborns and the babies.

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