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Research Article

Laboratory Detection of *Bordetella pertussis*: Are the Household Contacts a Major Sources of Infection for Infants?

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Keywords Pertussis; Sources of Infection; Infants; Household Contacts; Pulsed-Field Gel Electrophoresis; Serotyping

Abbreviations *B. pertussis: Bordetella pertussis;* WHO: World Health Organization; aP: Acellular Pertussis Vaccine; DTP: Diphtheria-Tetanus-Pertussis Vaccine; D: Diphtheria; T: Tetanus; wP: Whole-Cell Pertussis Vaccine; Hib: *Haemophilus Influenzae* B; PFGE: Pulsed-Field Gel Electrophoresis; RL: Regan-Lowe; MLST: Multilocus Sequence Typing; MLVA: Multilocus Variable Number Tandem Repeat Analysis

Abstract

Bordetella pertussis is the causative agent of pertussis, an infectious disease highly communicable, with a secondary attack rate up to 90% among non immune household contacts. In Brazil there are few studies identifying infant pertussis sources. The aim of this study was to demonstrate a possible source of infection of B. pertussis among household contacts with infants confirmed with pertussis by laboratory criteria, using Pulsed-Field Gel Electrophoresis (PFGE) that allows the identification of strains which can be epidemiologically linked to them.

From November/2011 to May/2012, nasopharyngeal swabs were collected from infants (< 7 months) suspected of pertussis. A total of 97 index cases were confirmed pertussis by PCR and/or culture. Samples were collected from up to five household contacts of each index case totaling 353. The strains were subtyped by pulsed-field gel electrophoresis and serotyping.

A total of 97 index cases and theirs 28 household contacts had the pertussis diagnosis confirmed by culture and/or Real-Time PCR. Among them was possible to characterize five groups of index cases/household contacts linked according to the degree of relatedness and genetic profiles obtained by PFGE technique, indicating the parents as a probable source of transmission of the disease to infants. Accordingly to the serotypes, all the five groups presented an agreement among the results of the index cases and their household contacts.

Based on our available evidence, it can be assumed that parents were a possible source of infection for these infants under seven months of age.

Hence we suggest with this study that mothers and fathers still play an important role in transmitting this disease to unprotected infants and new strategies are necessary to prevent this important disease that represents a great threat to public health.

Introduction

Bordetella pertussis is the causative agent of pertussis or "whooping cough", an infectious disease highly communicable, with a secondary attack rate up to 90% among non immune household contacts. Pertussis vaccines substantially reduced the impact of the disease in regions where vaccination rates are high. But, in spite of this, according to World Health Organization - WHO, in 2008, 16 million cases still occurred in the world, of that 95% were in developing countries, causing the death of 195,000 children [1].

In the pre-vaccine era, pertussis morbidity and mortality were high. Vaccination with inactivated bacterial suspensions was introduced in the 50s and 60s in many countries, substantially reducing the circulation of pertussis. However, its use has been associated with several side effects and in order to mitigate these effects, several countries have introduced Acellular Vaccine (aP) in their immunization schedule [2].

In the last 20 years, the disease has resurged in many highly vaccinated populations and there has been an increase in the number of adolescent and adult cases reported in many countries of North America, Australia and Europe [3,4].

Pertussis resurgence is probably due to several factors including increased recognition of the disease in adolescents and adults; waning immunity in vaccinated individuals; better detection methods; increased awareness among physicians and genetic changes in circulating strains of

B. pertussis, occurring under selective vaccination pressure. The contribution of these factors probably differs between countries [4-8].

In Brazil, a vaccination program was established in 1968 with the Diphtheria-Tetanus-Pertussis (DTP) vaccine, using the formulation composed of Diphtheria (D) and Tetanus (T) toxoids, and Whole-Cell Pertussis (wP). Currently the Brazilian Ministry of Health has recommended the use of a pentavalent vaccine composed of DTP, hepatitis B, and *Haemophilus Influenzae* B (Hib) at ages 2, 4 and 6 months and two boosters of DTP, the first at 15 months and the second between the ages of 4 and 6 years old [9].

It is known that the protection conferred by natural infection or vaccination do not induce the long-lived immunity, decreasing over the time (approximately five to ten years after the last booster) [6,10]. The absence of regular boosters either in the form of vaccine boosters or natural exposure to *B. pertussis* has been suggested to explain the shift in the age of distribution of pertussis, so reinfections at older ages are common. Then, these older contacts are often the source of infection for infants, who are at greatest risk for morbidity and mortality. Studies show that waning immunity in adolescents and adults was identified as a significant factor in transmission to young infants and approximately 75% of pertussis-infected infants acquired the disease from household contacts, most often a parent [3,6,11-13].

Frequently it is difficult to identify the source of infection for infants with pertussis and the studies addressing this setting like seroprevalence, surveillance, outbreaks, and the etiology of prolonged cough illness are required [12]. In Brazil there are few studies identifying infant pertussis sources [14-16] and the aim of this study was to demonstrate a possible source of infection of *B. pertussis* among household contacts (symptomatic or asymptomatic) to infants confirmed with pertussis by laboratory criteria (culture and/or Real-Time PCR), using Pulsed-Field Gel Electrophoresis (PFGE) that allows the identification of strains which can be epidemiologically linked.

Materials and Methods

Material collection

From November/2011 to May/2012, nasopharyngeal swabs were collected from infants (< 7 months) suspected of pertussis, regardless of vaccination status and included as criteria the presence of at least 10 days of coughing and one the following symptoms: paroxysmal coughing, post tussive vomiting, inspiratory “whooping”, cyanosis, apnea and choking.

The nasopharyngeal samples were collected with ultrathin and flexible sterile swabs and transported in Regan-Lowe (RL, Oxoid) charcoal semi-solid agar, supplemented with 10% sheep blood and 40 µg/mL cephalexin [17]. These samples were sent to the National Reference Laboratory for Pertussis, Instituto Adolfo Lutz, São Paulo, Brazil, to be analyzed for pertussis by culture and Real-Time PCR.

A total of 97 index cases in infants with pertussis confirmed by Real-Time PCR and/or culture were identified through the Pertussis Sentinel Network units of the São Paulo State, Brazil. Their household contacts were contacted by the investigators and invited to participate in the study that included an interview and collection of a nasopharyngeal sample by a trained investigator nurse. The interview was performed using a standard questionnaire on socio demographic characteristics, pertussis vaccine history, the presence of respiratory symptoms, recent treatment with antibiotics and habits. All interviews and material collections were performed after the signature of the informed consent form, under the coordination of professionals working at the Epidemiological Surveillance Center of São Paulo State Health Department.

Nasopharyngeal swabs were collected from up to five household contacts of each index case, regardless of the presence of any symptom, totaling 353 and were as follows: mother (n=76); father (n=62); siblings (n=54); grandparents (n=63) and others (n=96).

Culture and serotyping

The nasopharyngeal swabs were cultured on the same collection day on RL agar with 10% sheep blood and 40µg/mL cephalexin, and incubated at 35-37 °C under an ambient air with high humidity up to ten days. Colonies suggestive of the genus *Bordetella* were confirmed by Gram staining and oxidase reaction and species were identified by biochemical tests described previously [18]. The detection of the species-specific antigen O1 serotype and fimbrial antigens Fim 2 and Fim 3 were done by slide agglutination test using O1, and Fim 2 and Fim 3 antibodies, respectively. The O1 antiserum and fimbrial 2 and fimbrial 3 antibodies were produced at Instituto Adolfo Lutz.

DNA methods

DNA extraction from clinical specimens was performed using the MagNA Pure LC equipment using MagNA Pure LC DNA Isolation Kit I (Roche Applied Science, Indianapolis, IN) following the manufacturer´s instructions.

Real-Time PCR

We used the method described by Leite, et al. 2013. Real–Time PCR reaction was performed in the thermal cycler model LightCycler® 480 Software release 1.5.0 SP3 - Roche®, including specific primers and probes for detection of toxin gene *ptxS1* (GenBank accession n° AJ920066) and the insertion element IS*481* (GenBank accession n°M22031), present in multiple copies in the *B. pertussis*.

Pulsed-Field Gel Electrophoresis

We followed the PFGE method as described by Advani, et al. 2004, using *XbaI* as a restriction enzyme, with modifications as follows. Electrophoresis was performed at 6V/cm for 24 hours at 14 °C and pulse times of 5 to 6 s for 11h and pulse times of 8 to 35 s for 13h. The PFGE patterns were analyzed using BioNumerics software package (ver. 4.0; Applied Maths, Inc., Austin, TX, USA) and the similarity index was determined by the Dice coefficient of similarity.

Ethical aspects-The study followed recommendations provided by Resolution no. 466 of 2012 – National Health Council for Clinical Research in Humans and was approved by the Independent Ethics Committee of Irmandade da Santa Casa de São Paulo under the number 199/11. Protocol number 002/2012 – CEPIAL number 035/2011.

Patients or their representatives’ consents according to the informed consent form.

Results

A total of 97 index cases and their 28 household contacts have the pertussis diagnosis confirmed by culture and/or Real-Time PCR.

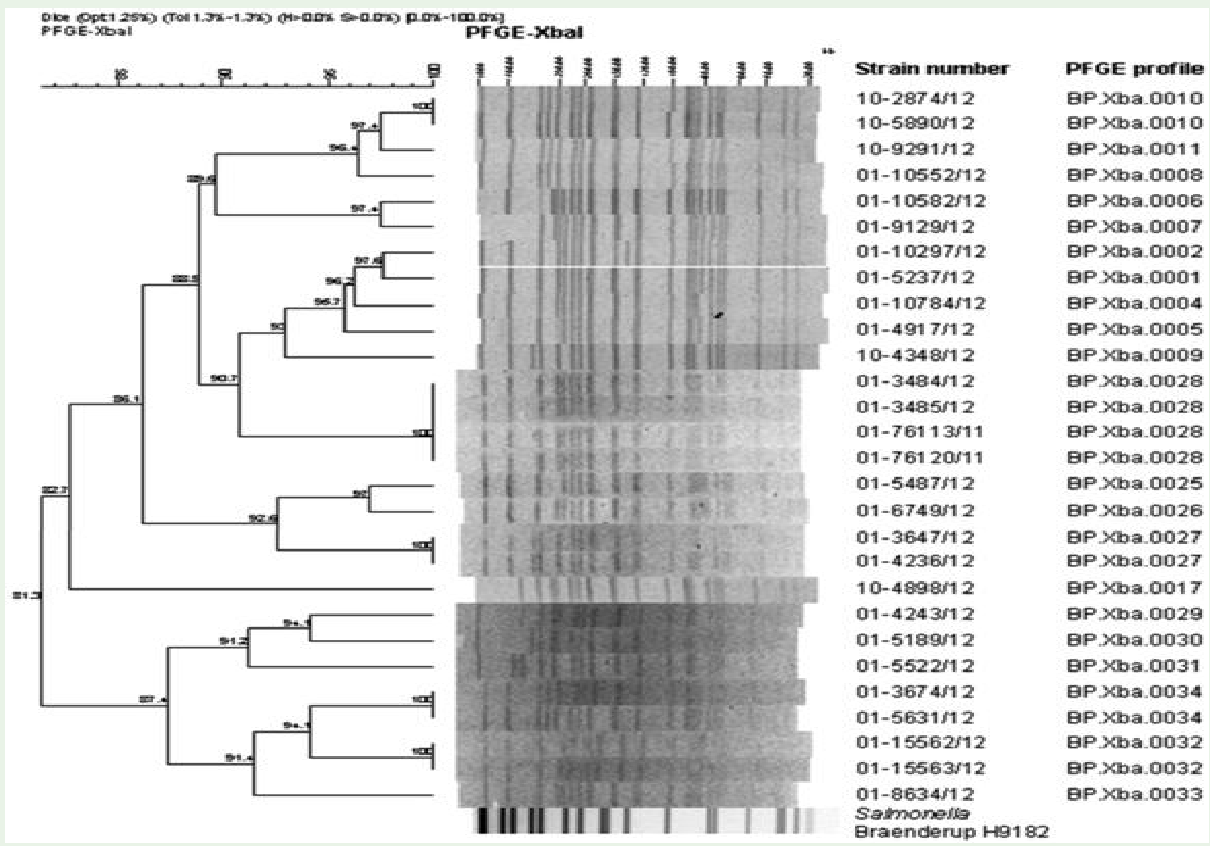

Figure 1: Relatedness of PFGE profiles of *B. pertussis* strains isolated from index case and household contacts.

Among them, 41 were confirmed positive for pertussis only by culture and 28 of them were serotyped and characterized by PFGE (13 were unviable after replating). The predominant serotype was Fim3 (22/28 78.5%), followed by Fim2, 3 (5/28 18.0%) and Fim2 (1/28 3.5%).

PFGE was highly discriminatory and among 28 strains analyzed, 21 PFGE profiles were identified, according to the nomenclature given by the Instituto Adolfo Lutz, with little genetic difference between them. Profile BP Xba0028 (14%, 04/28) was the most common type followed by BP Xba0010 (7%, 02/28), BP Xba0027 (7%, 02/28), BPXba0032 (7%, 02/28) and BP Xba0034 (7%, 02/28). Sixteen other profiles were identified and accounted for 57% of the isolates (Figure 1).

As shown in Table 1, it was possible to characterize five groups of index cases / household contacts (A, B, C, D and E) between the 97 index cases and 28 household contacts diagnosed positive by culture or Real-Time PCR. As can be seen, the five groups were connected according to the degree of relatedness and genetic profiles obtained by PFGE technique. By this methodology it was possible to identify the same genetic profiles of six household contacts from four index cases (B, C, D and E), indicating the parents as a probable source of transmission of the disease to infants. Only one index case with his household contacts (A) had a little genetic difference between them demonstrating the relatively homogeneous or clonal structure of *B. pertussis*. It was interesting to note that the group A, where there was a little genetic difference observed by PFGE, was precisely the group in which the grandmother was the probable source of infection, unlike the parents as was observed in the other four groups that presented same genetic profiles (B, C, D and E). Accordingly to the serotypes, all the five groups were in accordance with the results of the index cases and their household contacts.

Table 1: Profiles of serotype and PFGE type of *Bordetella pertussis* strains confirmed by laboratory criteria isolated from index case and their households.

	Patient	Culture	Real-Time PCR	Serotype	PFGE type
(A)	Index case	POSITIVE	POSITIVE	Fim3	BP.Xba.0025
	Household contact (grandmother)	POSITIVE	NEGATIVE	Fim3	BP.Xba.0026
(B)	Index case	NEGATIVE	POSITIVE	-	-
	Household contact (father)	POSITIVE	NEGATIVE	Fim2,3	BP.Xba.0028
	Household contact (mother)	POSITIVE	NEGATIVE	Fim2,3	BP.Xba.0028
(C)	Index case	NEGATIVE	POSITIVE	-	-
	Household contact (father)	POSITIVE	POSITIVE	Fim3	BP.Xba.0028
	Household contact (mother)	POSITIVE	POSITIVE	Fim3	BP.Xba.0028
(D)	Index case	POSITIVE	POSITIVE	Fim3	BP.Xba.0027
	Household contact (father)	POSITIVE	POSITIVE	Fim3	BP.Xba.0027
(E)	Index case	POSITIVE	POSITIVE	Fim3	BP.Xba.0032
	Household contact (mother)	POSITIVE	POSITIVE	Fim3	BP.Xba.0032

Discussion

Pertussis has re-emerged worldwide as an important cause of morbidity and mortality in infants especially during the first two months of life before pertussis immunizations begins. Several studies have evaluated the source of pertussis transmission to infants and typically report that pertussis infection results from the household contacts. Since 2002, numerous reports published from the United States, Canada, Europe, Australia, and China indicate the vulnerability in teenagers and young adults. Waning immunity following vaccination or natural infection is clearly documented and this waning in adolescents and adults who often have mild or atypical illness is recognized as an important reservoir of pertussis and as a significant source in transmission to young infants [3,5,8,13].

Another important cause for resurgence of pertussis is the vaccine-resistant strains, and many studies are investigating using molecular typing methods [5]. Several methodologies have been used for studying the molecular epidemiology of *B. pertussis* including serotyping, PFGE, Multilocus Sequence Typing (MLST), and Multilocus Variable Number Tandem Repeat Analysis (MLVA) [21]. PFGE analysis is used by many laboratories worldwide, however it is not possible to compare the genetic profiles isolated and tested with different protocols and nomenclatures [22]. This test is very useful to monitor the strains in circulation, for the detection of genetic changes and emergence of new strains.

In this study we could observe the epidemiological linkage of household contacts and index cases by the PFGE technique, suggesting that this group, in this case, the parents, were the probable source of transmission of pertussis to young infants. These findings are consistent with others studies that showed that household contacts, mainly the parents, are the most important source of infections of infants [16,23,24].

The parents or siblings are the most commonly identified source of infection and the mothers have been the most commonly mentioned source of infection in approximately twice as much as fathers. Although recent studies from Australia and the Netherlands have suggested that siblings are playing an important role in the transmission chain of pertussis to babies in these countries [8,13].

However it is often difficult to identify a source of infection in infants with pertussis because infection in adolescents and older adults can be difficult to diagnose due the symptoms that have been often atypical in this group and the nasopharyngeal cultures are rarely found to be positive [25,26]. The culture, although recognized as a highly specific method, presents a variable sensitivity, depending on many factors like a collection, transport and processing of the nasopharyngeal swabs. When the material collection is performed at the onset of the clinical manifestations high rates of *B. pertussis* isolation are achieved, but the positivity rate of culture decreases to about 15-20% with nasopharyngeal secretion collected three or more weeks after the onset of symptoms [19]. People treated with antibiotics or previously immunized also contribute to the low isolation rates by culture.

In our study, these factors contributed to the small number of samples confirmed by culture and most of them were confirmed by Real-Time PCR. Serology could not be included in this study and if it would be possible, probably the positivity rate among the communicants would be higher. Studies including the serology to investigate the sources of pertussis identified the source to children <6 months of age in about 50% of the contacts and similar studies have revealed a very high circulation of *B. pertussis* among adolescents and adults in vaccinated populations, with estimated yearly infection frequencies varying between 1% and 9% [4,23,27].

Regarding the results obtained by serotyping we could observe the predominance of serotype Fim3 which corroborates to previous studies [18,28] in which this predominance was also observed, suggesting that this serotype has been prevalent in this country for decades. The monitoring of circulating strains by serotyping is important for the detection of possible changes in *B. pertussis* in the population, since the fimbriae Fim2 and Fim3 are important vaccine components.

Conclusion

There were some limitations to this study, such as the small number of samples that were diagnosed positive by the culture technique. By this methodology it was only possible to identify seven household contacts from five index cases; and serological tests were not available to confirm pertussis.

The PFGE analysis could demonstrate in this study, the epidemiological linkage between index cases and household contacts. Based on our available evidence, it can be assumed that parents were possible sources of infection for these infants under seven months of age. Hence we suggest with this study that mothers and fathers still play an important role in transmitting this disease to unprotected infants and new strategies are necessary to prevent this important disease that represents a great threat to public health.

This information is essential to reinforce pertussis vaccination policy for pregnant women which are recommended by the Brazilian Ministry of Health. This adult Tdap vaccine should be given at every pregnancy from the 27th to 36th weeks of pregnancy. Another recommendation to protect young infants from pertussis is the vaccination of health professionals working in maternity and neonatal inpatient units [29].

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