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RARE DETECTION OF BORDETELLA PERTUSSIS-PERTACTIN DEFICIENT STRAINS IN ARGENTINA, A COUNTRY THAT USES THE WHOLE-CELL VACCINE FOR PRIMARY VACCINATION SERIES

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	detected in infants under-one-year-old. From the total analyzed B. pertussis isolates, 90.6% (317/350) contained the ptxP3-ptxA1-prn2-fim3-2 allelic profile. Only two pertactin-deficient isolates were found by immunoblotting and sequencing techniques. The low prevalence of pertactin-deficient-strains detected in Argentina would suggest that the loss of pertactin-gene expression might be aP-vaccine-driven.

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Ref. //. " *Rare detection of Bordetella pertussis-pertactin deficient strains in Argentina, a country that uses the whole-cell vaccine for primary vaccination series"* by Carriquiriborde et al. submitted for publication in Emerging Infectious Diseases

Dear Editor

We would like to thank the editor and reviewer involved for sharing their time, attention and experience to review the manuscript [EID-19-0329]. All comments have helped to improve the manuscript. We have reduced and focused the manuscript in order to clarify the message discussing the PRN deficient strains in Argentina. We have deleted various paragraphs following the suggestions (not shown in the new version of the manuscript) and the suggested text modifications have been introduced (highlighted in yellow) in this revised version of the manuscript. Headings in the Methods and the Results were incorporated. The vaccine schedule was moved from Introduction to the Methods. We clarified the index of discrimination and resubmitted the figure 1 panels A and B as separate files.

We wish to resubmit "*Rare detection of Bordetella pertussis-pertactin deficient strains in Argentina, a country that uses the whole-cell vaccine for primary vaccination series*", for further consideration and publication in Emerging Infectious Diseases.

We look forward to your reply.

Sincerely,

Dra. Daniela Hozbor

RARE DETECTION OF BORDETELLA PERTUSSIS-PERTACTIN DEFICIENT STRAINS IN ARGENTINA, A COUNTRY THAT USES THE WHOLE-CELL VACCINE FOR PRIMARY VACCINATION SERIES

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ABSTRACT

Pertussis resurgence had been attributed to waning vaccine immunity and *Bordetella pertussis* adaptation to escape vaccine-induced immunity. Circulating bacteria differ genotypically from strains used in pertussis-vaccine production. Pertactin-deficient-strains are highly prevalent in aP-vaccinating countries, suggesting strong aP-imposed selection of the circulating bacteria. To corroborate this hypothesis, systematic studies on PRN-prevalence performed in wP-using countries are needed. We present pertussis-epidemiological data and molecular characterization of *B. pertussis* isolates obtained during 2000-2017 in Buenos Aires, a wP-primary-vaccination-employing area. From 2002 pertussis-case incidences increased with regular 4-year outbreaks, with most cases detected in infants under-one-year-old. From the total analyzed *B. pertussis* isolates, 90.6% (317/350) contained the *ptx*P3-*ptx*A1-*prn*2-*fim*3-2 allelic profile. Only two pertactin-deficient isolates were found by immunoblotting and sequencing techniques. The low prevalence of pertactin-deficient-strains detected in Argentina would suggest that the loss of pertactin-gene expression might be aP-vaccine-driven.

INTRODUCTION

Vaccination against pertussis is mandatory worldwide. Two types of vaccines are currently in use: whole cell vaccine (wP), which was the first vaccine developed and acellular vaccine (aP) containing purified components of *Bordetella pertussis*, formulated subsequently due to the adverse reactions associated with the wP (1).

Many countries continued using wP for the primary vaccination series and for the boosters recommended for children under 7 years of age. The industrialized countries have switched to aP vaccination. In the last two decades, however, the number of pertussis cases detected increased to around 24.1 million per year, with approximately 161,000 deaths (2, 3). Though most cases occur in developing, developed countries have also had large-scale outbreaks, even those nations with high vaccination rates (2, 4-6).

The main causes proposed for this worrisome pertussis epidemiology are: vaccination coverages lower than the >90% recommended by the World Health Organization (WHO), a waning of the vaccine-induced immunity (7, 8)—occurring faster in the acellular vaccinated population—and an evolution of the circulating bacteria to vaccine-immunity-evasive phenotypes (9, 10). The first reports on bacterial evolution documented genetic polymorphisms encoding the proteins included in the vaccines—*e. g.*, pertactin (PRN) and pertussis toxin—and later in the pertussis-toxin promoter (*ptx*P) (11, 12). More recently, a major increase in the isolation of *B. pertussis* bacteria that do not express certain vaccine antigens was reported (10, 13, 14). In countries using PRN-containing aP vaccines like the USA, Canada, and Australia, the PRN-deficient isolates increased substantially in the last 4 years (10, 15, 16). The expansion of strains deficient in PRN in populations vaccinated with PRN-containing aP vaccines indicates that such strains apparently have a selective advantage in aP-vaccinated populations (17). To corroborate this hypothesis, we undertook systematic studies on PRN prevalence in Argentina, a wP-using country. We monitored and analyzed the *B. pertussis*–population dynamics in Buenos Aires (Argentina). Our aim was to assess whether or not PRN-deficient strains were circulating in Buenos Aires and to analyze the results obtained in relation

to the vaccine used and the epidemiological situation of the disease in 2000-2017 period.

MATERIALS AND METHODS

Population studied, clinical-case definition, and laboratory diagnosis

We used pertussis epidemiological data and samples collected during 2000–2017 from the Pertussis Reference Laboratory in La Plata (Laboratorio VacSal. Instituto de Biotecnología y Biología Molecular. Facultad de Ciencias Exactas, Universidad Nacional de La Plata, CONICET La Plata, Buenos Aires). Data on gender, age, duration of symptoms, vaccination status, and laboratory results were collected.

Pertussis clinical-case was confirmed in patients by *B. pertussis* isolation in culture, amplification of *B. pertussis*-specific DNA by PCR, or serology result of pertussis toxin (PT) immunoglobulin G (IgG) >120 IU/mL. A confirmed case of pertussis is also defined as a case that meets the clinical case definition and is epidemiologically linked to a laboratory confirmed case (18, 19) (20).

Vaccine schedule used in Buenos Aires

The wP was introduced in Argentina—a country of 44.9 million inhabitants—in the 1970s and is still in use for the three primary doses at 2, 4, and 6 months and for the two boosters at 18 months and school entry at 5–6 years in the public sector (around 90% of the population). The aP vaccine is used in the private sector and for the boosters in adolescents, healthcare workers in contact with infants under 12 months, household contacts of very-low-birth-weight infants, and during pregnancies.

Though in most of Argentina, the DTP3 (diphteria-tetanus-pertussis–containing vaccine as a third dose) coverage during recent years ranged between 91.0% and 95.0%, in certain jurisdictions that figure was 80.0% or lower (21). The

official coverages for adolescent boosters and maternal-immunization for 2015, 2016, and 2017 were 75.3, 81.9%, and 88.0%, and 61.7%, 65.6%, and 67.0%, respectively.

Samples and bacterial-growth conditions

The Pertussis Reference Laboratory samples included nasopharyngeal specimens from 16,151 hospitalized patients from Buenos Aires with signs of pertussis infection. These samples were routinely screened for *B. pertussis* by culture and PCR. *B. pertussis* culture was performed on Regan-Lowe agar (Difco) supplemented with 15% (v/v) defibrinated fresh sheep blood at 36.5 °C and monitored for 10 days. Suspected colonies were replicated in Bordet-Gengou agar (Difco) supplemented with 15% (v/v) defibrinated fresh sheep blood. Colonies exhibiting hemolysis were Gram-stained and tested by agglutination with *B. pertussis*–specific antiserum (Murex Diagnostic, Dartfort, England) and PCR (22, 23). The isolates were also biochemically typed by the API-20-NE system (bioMérieux, Marcy l'Étoile, France).

The isolates were stored at –80 °C in 1% (w/v) Casaminoacid solution containing 20% (v/v) glycerol. *B. pertussis* strain Tohama phase I (Collection de l'Institut Pasteur) was also grown on Bordet-Gengou agar at 36.5 ºC for 72 h.

***B. pertussis*–isolate characterization**

Genotyping

Total *B. pertussis* isolates (n=350) collected in Buenos Aires from January 2000 through December 2017 were included in the analyses (Table 1).

For genotypification, the pertussis-toxin-promoter (*ptx*P), pertussis-toxin-A-subunit (*ptx*A), PRN (*prn*), and fimbriae-type-3 (*fim*3) loci were PCR-amplified with the respective primers indicated in Table 2 and sequenced as previously described (24-32). The isolates were also screened for an array of mutations causing deficiency in the immunogen PRN through PCR amplification and molecular sequencing (26, 29). Primers CCCATTCTTCCCTGTTCC AT and GCCTGAGCCTGGAGACTGG (26) were used to amplify the complete *prn* gene (26). These primers in combination with internal primers were used to sequence the complete gene.

The discriminatory power of the MLST technique here used was calculated by year using the equation reported by Hunter et al (33). This equation is based on the probability that two unrelated strains sampled from the test population will be placed into different typing groups. Thus, the index can take any value between 0 and 1, the former representing the lowest discriminatory capacity meaning that all the strains being in a single genotyping group (lowest diversity) and the latter representing the largest discriminatory capacity indicating high genotypic diversity among the isolates.

PRN immunoblotting

For this assay, 2 **x**10^{10} colony-forming units of *B. pertussis* isolates were treated with Laemmli sample buffer and the extracts run on 12.5% (w/v) sodium-dodecylsulfate-polyacrylamide gels. After electrophoresis, the proteins were transferred from the polyacrylamide to a polyvinylidenphosphate membrane (Immobilon P, Millipore) and were incubated with a 1:2,500 dilution of PRN-specific polyclonal immune sera. This was obtained from BALB/c mice immunized with purified *B. pertussis* 69-kDa PRN

(NIBSC Code Nº 90/654 version 4). Alkaline-phosphatase-labelled sheep anti(mouse immunoglobulins) was used for detecting the immune complexes. Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were the phosphatase chromogenic substrates (Biodynamics SRL, Buenos Aires, Argentina). *B. pertussis* Tohama strain was served as the PRN-positive control.

Serotype analysis

Serotype analysis was also performed using an agglutination assay with monoclonal antibodies against fimbriae type 2 (anti-Fim2 mAb; NIBSC 04/154) and fimbriae type 3 (anti-Fim3 mAb, NIBSC, 04/156) according to the EU laboratory group recommendations (34).

RESULTS

Pertussis epidemiology in Buenos Aires

In this section we describe the epidemiology of pertussis in Buenos Aires (the most populated province of Argentina) during 2000–2017. During these years, the Pertussis Reference Laboratory located at La Plata city received 75% of the total clinical samples (nasopharyngeal samples) from pertussis-suspected cases detected in Buenos Aires and reported to the Ministry of Health, including a total of 16,151 samples from which 3,220 (19.9%) were laboratory-confirmed cases. Two thousand and eight hundred seventy samples were positive by PCR for *B.* pertussis-specific genes and 350 samples were positive by PCR and culture.

The provincial cases-per-year distribution reflected the pattern of the whole country with the three outbreaks detected, in 2008, 2011, and 2016 (Fig. 1 Panels A and B). In each year of the period analyzed, most of the cases were detected in the

groups of 0-to-2-month (m)– and 2-to-4-month–old infants (Fig. 2). The high proportion of cases recorded in patients younger than 6 months was expected since pertussis is most severe in that age group.

Regarding the distribution of confirmed-pertussis-case according to patient-age and vaccination status, of the confirmed cases, in 72.6% (2338/3220) those data were complete, with 26.5% (619/2338) non-vaccinated because of their age that was lower than 2 months. Of the total infants with less than 6 m, 45.3% received complete age-specific vaccination schedules, respectively. The percentage of patients with complete schedules for children above 6 months was 53.7% and for adolescents above 11 years only 6.4%. Though this last percentage is low, this age group contained considerably fewer individuals than those below age 6 months (44 individuals *vs*. 1,590 children above 6 months).

B. pertussis genotyping

Almost all *B. pertussis* isolates analyzed contained the *ptxA1* (99.7%) and *prn2* (98.8%) alleles. The clinical isolates obtained during 2000-2004 period harbored up to 4 different MLST genotypes (Fig. 3). The index of discrimination calculated by year for this period ranged from 0.25 to 0.80. The highest value (higher diversity) was detected in 2000. The *ptx*P1 or *ptx*P4 variant was detected before 2004; thereafter the *ptx*P3 locus prevailed. The majority of the isolates obtained after 2004 were of the *ptx*P3-*ptx*A1-*prn*2-*fim*3-2 genotype (291/350, 83.1%). For the 2004-2017 period, the index of discrimination ranged from 0 to 0.24, indicating the lowest diversity detected.

Fimbriae serotyping

From the total tested isolates (n=350) only 1 obtained in 2016 was classified as Fim2 whereas the rest of the isolates were Fim3.

PRN Immunoblots

Only two of the total *B. pertussis* isolates included in this study were PRN-deficient. Both strains were obtained from patients below age 1 year with typical pertussis symptoms. These cases were linked in time (2016) but not geographically. One of these patients was born to mother vaccinated with a PRN-containing aP vaccine and the other to non-vaccinated mother. For these two strains we detected IS481 sequence (in fw sense) at position 1613-1614 of *prn* disrupting the gene.

DISCUSSION

We undertook a molecular-genetic characterization of the total *B. pertussis* isolates (n=350) obtained during 2000-2017 period from hospitalized patients in Buenos Aires, Argentina. Buenos Aires as the whole country uses only wP for primary series of pertussis vaccinations. The majority of *B. pertussis* isolates were obtained during the outbreaks detected in 2007-2008 (n=83); 2011-2012 (n=145) and in 2016-2017 (n=45). Seventy eight percent of the total isolates became from patients with less than 6 months of age, 13,7% from patients with ages that range from 6 to 12 months and 8.3% from patients with > 12 months. As expected the majority of the *B. pertussis* isolates became from unvaccinated individuals because of the age or incomplete vaccinated for age. As was detected in other countries, almost all isolates here characterized were classified as Fim3 serotype (35).

Of the total 350 isolates, the variants *ptxP1* and *ptxP*4, and the allele *prn*1 were detected before 2004. After 2004, the total isolates obtained (n=313) carried

194 *ptxP3-ptxA1-prn2* alleles with the *fim3-1* or *fim3-2* combination. These genotypes
195 differed from those of the vaccine-production strains (36) and were the most common
196 in other countries that were highly vaccinated (35).
197 The polymorphism in PRN first described and the subsequent spread of PRN-
198 deficient isolates have elicited a deep concern in the healthcare system since these
199 changes hypothetically might represent a selective avoidance by the bacteria of the
200 immunity induced by the vaccines. The *prn*2 predominance detected in the more
201 recent Buenos-Aires isolates agrees with the hypothesis that strains in the vaccinated
202 population with that allele are fitter than those harboring other *prn* alleles (37).
203 As to a deficiency in PRN expression, we detected only two isolates
204 containing an IS481 in the coding region of *prn*. These isolates were obtained from
205 patients with less than 1 year of age linked in time (obtained in 2016) but not in place.
206 One of these patients was born to a mother vaccinated with a PRN-containing aP
207 vaccine and the other to non-vaccinated mother.
208 We were interested to note that we had previously received practically no
209 reports on those details in a country like Argentina where wP is the only vaccine
210 included in the calendar for primary pertussis vaccinations. Recently, Poland—the
211 only country in Europe that still uses the wP but also the aP vaccine for primary
212 series—has reported the detection of PRN-deficient clinical isolates (38). The
213 percentage of those isolates was lower (15%) than that detected in USA, Canada or
214 Australia where use only the aP vaccines (>65%; (10, 39, 40). The authors of such
215 article suggested that the detection of those isolates might be a consequence of the
216 increase in the use of aP vaccines in Poland. Within this context, our work is
217 apparently unique since Argentina uses only wP vaccine for the primary series of
218 pertussis vaccinations.

This low frequency of PRN-deficient strains in regions where wP is still in use supports the proposed hypothesis that PRN-deficient clinical isolates present an advantage in an aP-vaccine–primed immunity (41). Accordingly, PRN-deficient clinical isolates were able to overcome an anti-PRN–mediated inhibition of macrophage cytotoxicity *in vitro* (42). Moreover, a recent study revealed that recently collected PRN-deficient *B. pertussis* clinical isolates harboring a *ptx*P3 variant and the *prn*2 allele remained at higher colony-forming units/lung and were capable of sustaining infection longer in aP-immunized mice than isolates still producing the protein (42). The authors of that study speculated that these particular isolates might thus be capable of infecting immunized individuals at an earlier stage of waning immunity after aP-vaccine immunization or post-infection, thus having an advantage over isolates producing PRN. These findings of Hegerle et al. (42) are consistent with those recently reported by Safarchi et al. (17) indicating a higher fitness of PRN-negative strains in aP-immunized mice. These latter authors demonstrated in a mixed-infection model in which PRN-negative *B. pertussis* colonized the respiratory tract of aP-immunized mice more effectively than the PRN-positive strain, thus outcompeting that strain (17).

Regarding a possible association between clinical findings and the PRN expression of the bacterial isolates that caused the human infections; recent studies suggest that symptoms (with the exception of apneas which was less likely in PRN deficient infections) and clinical course were similar regardless PRN expression (14, 41). Clarke et al (2015) added new data on this subject that suggest that the rapid emergence of PRN deficient *B. pertussis* variants is unlikely to contribute to any greater risk of death or severe outcomes from infections in young, vulnerable infants (43).

Studies like the one reported here support the ongoing hypothesis regarding the pathogen adaptation of *B. pertussis* to the type of vaccine used. A key finding in this work was that the use of the wP in the primary series of vaccinations correlated with a near-complete absence of PRN-deficient strains even though the aP vaccine was employed in subsequent regimes. A continued surveillance for PRN production in circulating *B. pertussis* is needed, as well as a monitoring of other possible

genotypic changes in the *B. pertussis* population, including a lack of expression of other immunogens contained in acellular vaccines.

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Dr. Donald F. Haggerty, a retired academic career investigator and native English speaker, edited the final version of the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHORS AND CONTRIBUTORS

DFH planned the study, interpreted data, and wrote the manuscript. DB, planned the study, interpreted data, and edited the figures and manuscript. FC, VR, PMA, GM and EB performed experiments and laboratory analyses. All authors approved the final manuscript.

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Table 1: Immunization status of the patients infected with the *B. pertussis* strains studied

Year	Number of strains	Patient information						
		< 2 m of age	< 6 m of age		6-12 m of age		> 12 m of age	
		Unvaccinated because of age	Incomplete vaccination schedule	Complete vaccination schedule	Incomplete vaccination schedule	Complete vaccination schedule	Incomplete vaccination schedule	Complete vaccination schedule
2000	7	3	1	1	2			
2001	7	2	1	1	3			
2002	5	3	1	1				
2003	9	4	2	2	1			
2004	9	3	2	2	2			
2005	6	3	1	1	1			
2006	6		3		3			
2007	38	10	10	9	6	3		
2008	45	21	10	6	4		4	
2009	7	3	2	2				
2010	6	4	2					
2011	86	40	20	16	2	4		4
2012	59	20	15	10		9	5	
2013	6	3				3		
2014	3		2			1		
2015	6	3	1	1			1	
2016	32	12	1	8	2		1	8
2017	13	3	1	1		2		6

Complete Vaccination Schedule refers to that the individual according to its age received the total number of doses indicated in the National Vaccination Calendar.

Incomplete Vaccination Schedule refers to that the individual according to its age did not receive the total number of doses indicated in the National Vaccination Calendar

Table 2. Primers used in the polymerase-chain reaction

Gene	Primer Sequence	References
*ptx*P	F: 5'-AATCGTCCTGCTCAACCGCC-3' R: 5'-GGTATACGGTGGCGGGAGGA-3'	(27, 28)
*ptx*A	F: 5'-CCCCTGCCATGGTGTGATC-3' R: 5'-TCAATTACCGGAGTTGGGCG-3'	(29)
prn	F: 5'-CAATGTCACGGTCCAA-3' R: 5'-GCAAGGTGATCGACAGGG-3'	(26)
fim3	F: 5'-GACCTGATATTCTGATGCCG-3' R: 5'-AAGGCTTGCCGGTTTTTTTTGG-3'	(31)

LEGENDS TO THE FIGURES

Fig. 1. Panel A. Number of suspected-pertussis cases reported to the Reference Laboratory per year during 2000–2017 in the Buenos-Aires province. Panel B. Number of laboratory-positive pertussis cases during those same years. The numbers above the bars denote the precise *ordinate* values.

Fig. 2. Number of laboratory-positive pertussis cases according to age during 2000–2017 for the seven cohorts between ages 0 and >11 years (*cf.* key to bar textures). The age groups did not include the patients with ages on the border.

Fig. 3. Percentage of multi-locus-sequence-typing genotypes (*cf.* bar-texture key) among isolates collected between 2000 and 2017 in Buenos Aires, Argentina

Figure 1 Carriquiriborde et al

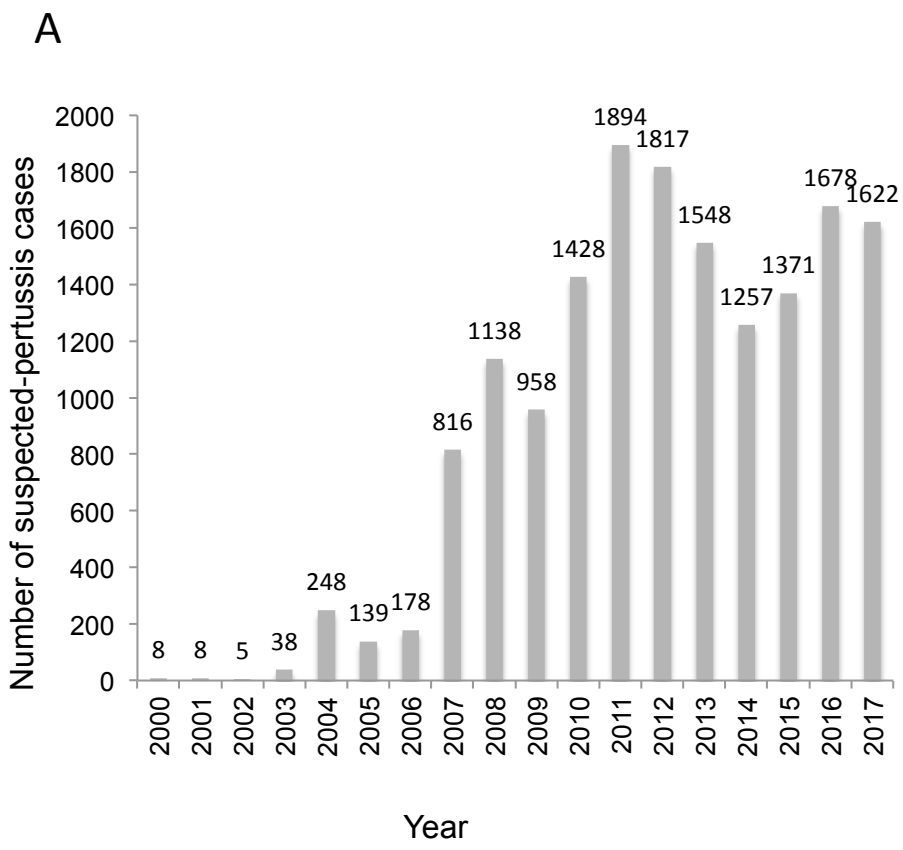
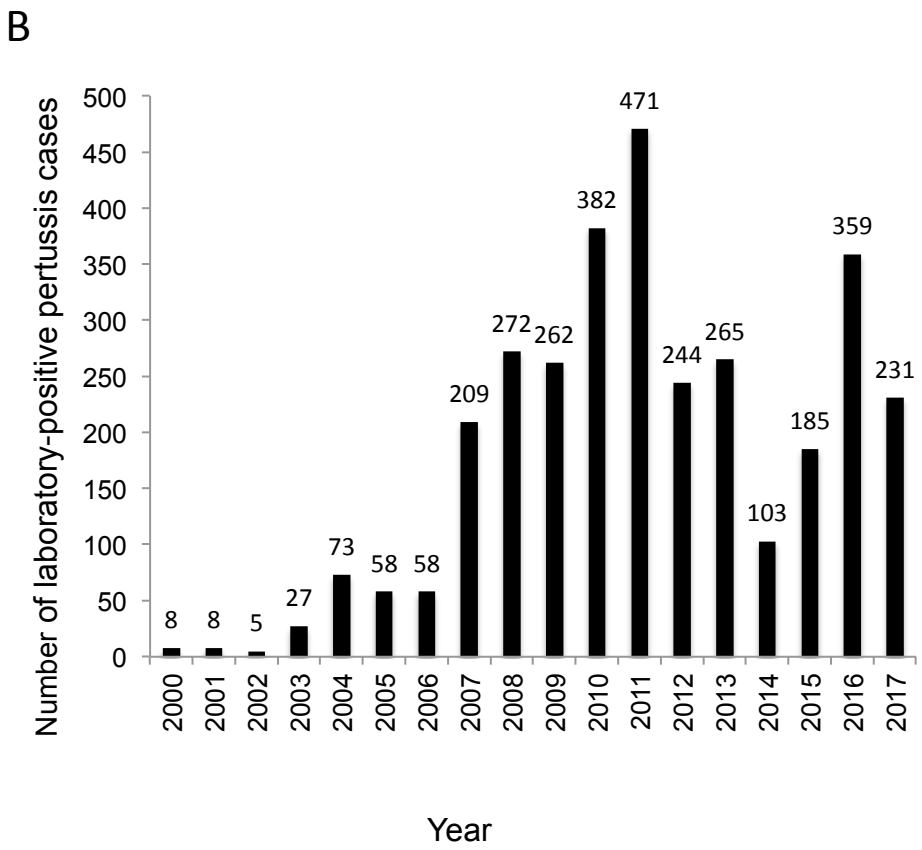


Figure 1 Carriquiriborde et al



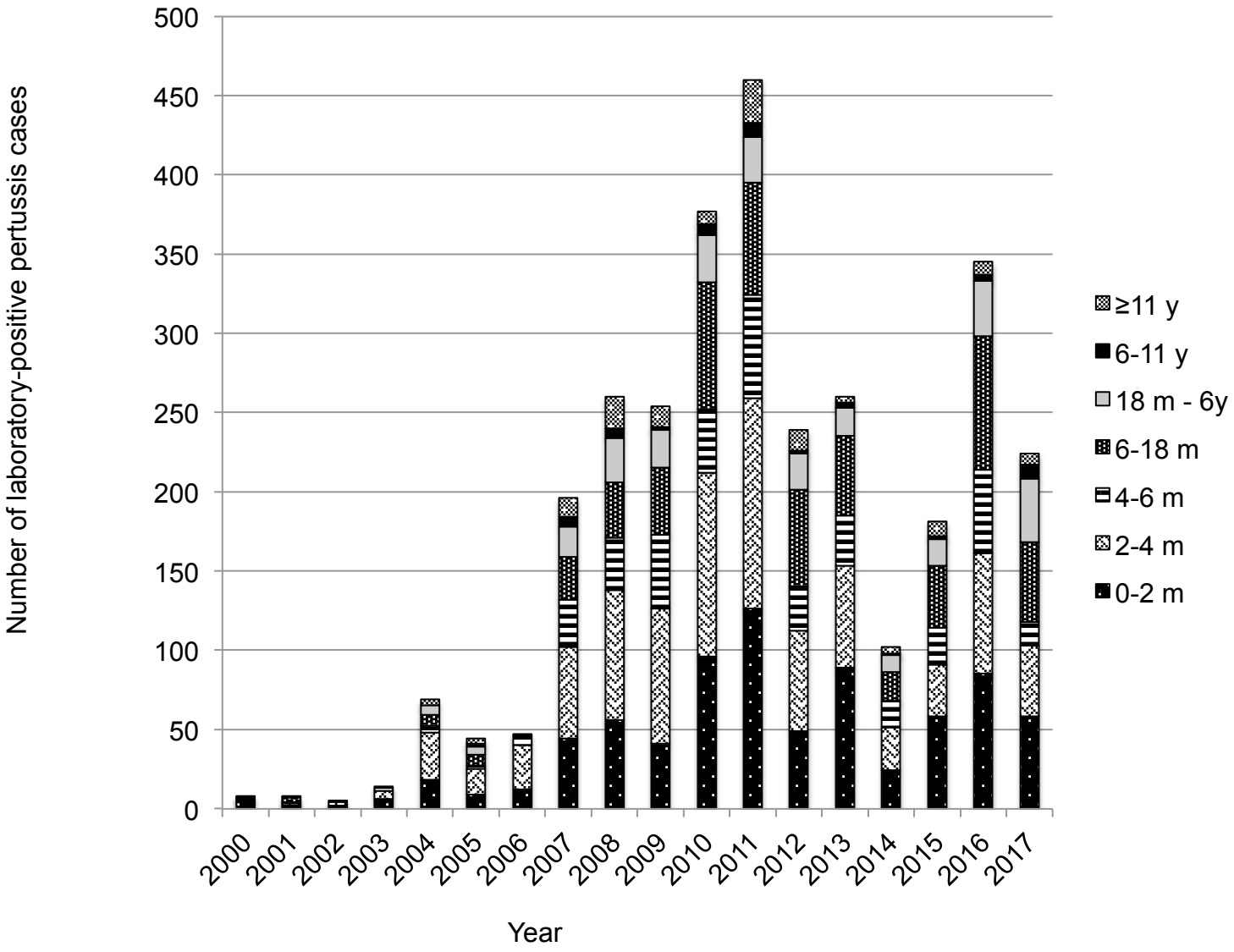
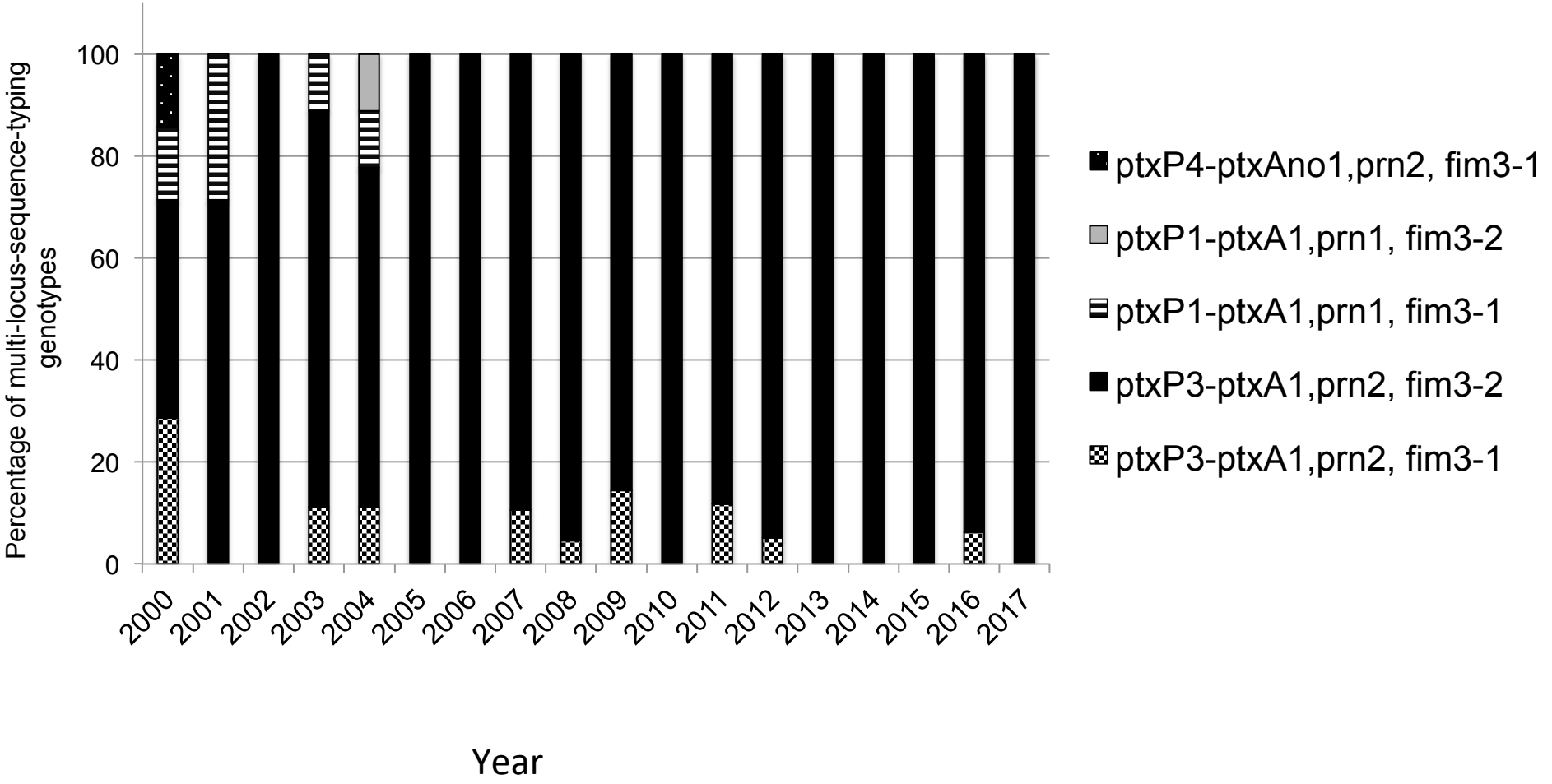

Number of laboratory-positive pertussis cases
500
450
400
350
300
250
200
150
100
50
0
2000
2001
2002
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
2014
2015
2016
2017
Year
≥11 y
6-11 y
18 m - 6y
6-18 m
4-6 m
2-4 m
0-2 m

Figure 3 Carriquiriborde et al



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