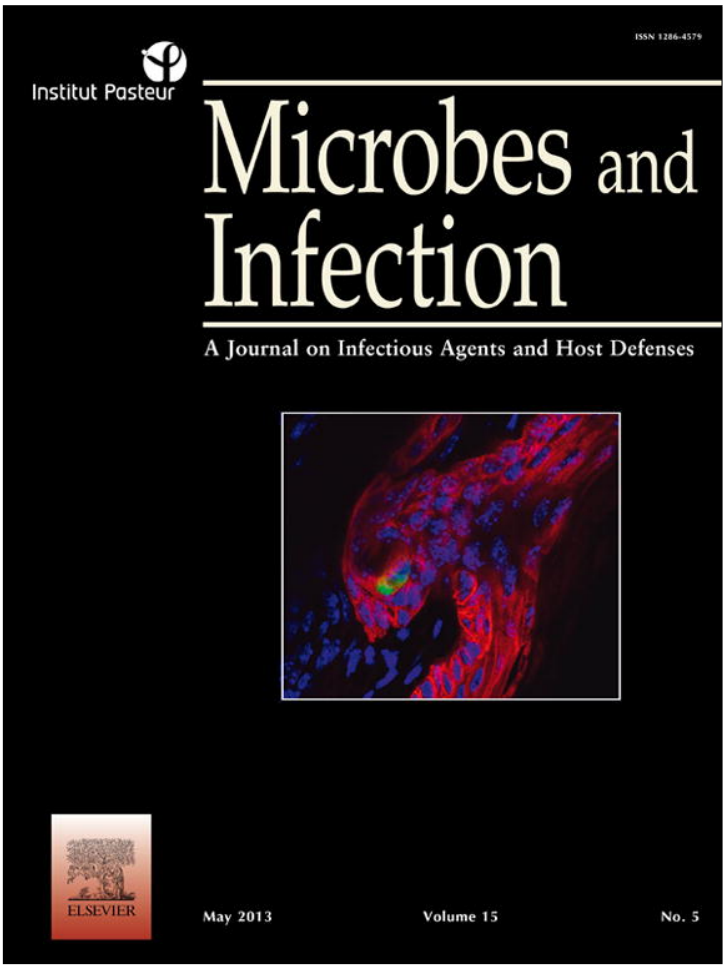

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Original article

In-vitro and *in-vivo* analysis of the production of the *Bordetella* type three secretion system effector A in *Bordetella pertussis*, *Bordetella parapertussis* and *Bordetella bronchiseptica*

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Abstract

Bordetella pertussis, *Bordetella parapertussis*, and *Bordetella bronchiseptica* are three closely related pathogens.

They all possess the gene coding for the *Bordetella* type three secretion system effector A (*bte*A) toxin that became a focus of interest since it was demonstrated that *B. pertussis* Japanese non-vaccine-type isolates produce BteA unlike vaccine-type isolates. We thus explored the *in-vitro* production of BteA in *B. pertussis* isolates collected in France during periods of different vaccine policy as well as in *B. parapertussis* and *B. bronchiseptica* isolates. We also analyzed the *in-vivo* induction of anti-BteA antibodies after infection with different isolates of the three species.

We produced a recombinant His6-tagged BteA (rBteA) protein. Specific rBteA polyclonal serum was prepared which enabled us to screen *Bordetella* isolates for *in-vitro* BteA production: 99.0% (293/296) of tested *B. pertussis* isolates, including French vaccine strains, and 97.5% (79/81) of *B. bronchiseptica* isolates produced BteA *in-vitro* but only the latter was capable of inducing an *in-vivo* immune response. No *in-vitro* or *in-vivo* production of BteA was detected by any of the *B. parapertussis* isolates tested.

Keywords: *Bordetella*; Vaccination; BteA

1. Introduction

Pertussis, or whooping cough, is a severe respiratory disease, particularly life-threatening for infants. Responsible for over 195 000 children deaths worldwide in 2008 according to WHO estimates, whooping cough is caused by a Gram negative bacterium called *Bordetella pertussis* [1]. Besides *B. pertussis*, *Bordetella parapertussis* is also responsible for a severe respiratory disease with pertussis-like symptoms [2]. These bacteria both belong to the *Bordetella* genus, share the same human host and have possibly emerged out of two distinct lineages of *Bordetella bronchiseptica* [3,4], another member of this genus capable of infecting a wide range of four legged mammals and humans [2].

To infect and colonize the respiratory tract of their hosts, *Bordetella* species display a wide array of virulence factors enabling their adherence to the respiratory epithelium and preventing their clearance by the host immune system. *B. pertussis* expresses adhesins, including filamentous hemagglutinin (FHA) and pertactin (PRN), which act as anchors for adherence, and toxins, notably pertussis toxin (PT) and adenylate cyclase-hemolysin toxin (AC-Hly), acting as immune modulators to prevent clearance by the immune system [2]. Being genetically related, *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* are also closely related at the proteomic level and thus have many virulence factors in common [2].

Studying the mechanisms of pathogenicity enabled the development of vaccines directed against *B. pertussis*.

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Whooping cough thus became a vaccine preventable disease in the 1950s with first, the introduction of whole cell pertussis (wP) vaccines targeting the whole bacteria and then, the introduction of acellular pertussis (aP) vaccines targeting specific virulence factors of *B. pertussis*. These vaccines have been used in effective vaccination programs leading to a substantial decrease in mortality and morbidity among infants.

The widespread use of wP vaccines resulted in a control of isolates similar to vaccine strains leading to a monomorphic population [5,6]. On the contrary, aP vaccines, containing only a small number of purified virulence factors, induce an immune response capable of targeting all virulent circulating isolates rather than a particular *B. pertussis* subpopulation. It has been hypothesized that a few years after the change of wP vaccine by aP vaccines, isolates not producing one or more virulence factors would be more likely to be selected [7]. Recent studies in Finland, France and Japan indicate that loss of PRN expression is the phenotype most frequently observed that might be linked to aP vaccine-induced immunity [8–11]. Nevertheless, *B. pertussis* and *B. parapertussis* still circulate and evolve in vaccinated populations [10]. Whereas most researches focus on evolution of vaccine-antigens, non-vaccine virulence factors gain interest for future trends in vaccination strategies.

Among these non-vaccine factors, the *Bordetella* Type Three Secretion System Effector A (BteA) protein is a toxin that has recently received more attention [12]. BteA is secreted through the *bvg*-regulated *bsp*-encoded Type Three Secretion System (TTSS), and is a non-apoptotic cytotoxic effector for a wide range of mammalian cells. First described in *B. bronchiseptica* after a genome-wide screening, *bte*A (Gene ID BB4228) encodes a predicted 68 kDa protein, BteA, involved in TTSS dependant cell lysis *in-vitro* [13].

Both *B. pertussis* and *B. parapertussis* carry the TTSS gene loci and *bte*A (Gene ID BP0500 and BPP3783 respectively) which are similarly transcribed and regulated in these two closely related species [14] and TTSS has been shown to contribute to *B. pertussis* infectious processes [15]. Expression of *bte*A and production of BteA have been demonstrated in *B. bronchiseptica* [13,16], and more recently in Japanese non-vaccine-type *B. pertussis* isolates [12] whereas no proof of *B. parapertussis* isolates producing BteA has been made yet.

bteA is shared by three, human-infecting, *Bordetella* species, is highly conserved (over 96% similarity) and BteA is not targeted by current vaccine-induced immunity. We produced polyclonal mouse antibodies directed against a recombinant-BteA (rBteA) protein, and used them to screen French *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* isolates by western blotting for production of BteA. The aim was to assess whether or not vaccine-induced herd immunity has affected the production of this toxin as suggested by Han and co-workers for Japanese isolates after wP vaccine introduction [12]. We also used a murine model of respiratory infection to investigate the possible *in-vivo* production of BteA by some *Bordetella* isolates during infection. Over 98% of tested *B. pertussis* and *B. bronchiseptica* isolates produced BteA *in-vitro* but only the latter was capable of inducing an *in-vivo* immune response. No production of this toxin was found in any tested *B. parapertussis* isolate.

2. Material and methods

2.1. Bacterial strains and isolates, and culture conditions

We used *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* reference strains Tohama, 12822 and RB50 respectively as well as French wP vaccine strains Bp1414 and Bp1416 [17]. We also used isolates collected during the pre-vaccine era [from the collection of the Institut Pasteur (CIP)], and isolates collected during the post-wP vaccine era and post-aP vaccine era (from the collection of the National Reference Centre) [9,10,17–19].

Bacteria were grown on Bordet-Gengou agar (BGA, Difco) supplemented with 15% defibrinated sheep blood at 36 °C for 72 h and plated again for 24 h (*B. pertussis* and *B. parapertussis*) or 16 h (*B. bronchiseptica*) before each experiment. For some experiments, low passage clinical isolates and the reference strain Tohama were subcultured five times on BGA plates. When necessary, isolates and reference strains were cultured in modified Stainer Scholte [20] medium until the OD_{650} reached 1 starting from 0.2 for *B. pertussis* and 0.05 for *B. bronchiseptica*.

For secretion analysis, selected isolates and reference strains were cultured in Stainer Scholte medium. BteA secretion was analyzed when OD_{650} reached 1 as well as after 24 h of culture. At these time points, the bacterial suspension was centrifuged 5 min at 10,000 rpm on a bench centrifuge and supernatant was filtered through a PVDF Millex 0.22 μm pore filter (Millipore) prior to concentration (40×) with a 10K Amicon Ultra (Millipore) according to the manufacturer's recommendations. The equivalent of 500 μL of supernatant was subjected to Western Blot analysis for BteA and other virulence factor secretion.

2.2. PCR amplification, cloning and production of recombinant His6-BteA (rBteA)

Primers BP0500-for (5′-AAAAAAGGATCCATGTTGAGCAACAACGTCAATCCGGTC-3′) and BP0500-rev (5′-AAAAAAAAGCTTTCATGCGCGTAGATTCAGCGCCGTGATC-3′) were used to amplify the *bte*A ORF (Gene ID BB4228) from *B. bronchiseptica* RB50. These primers allowed introduction of BamHI and HindIII restriction sites for manipulation of the PCR products. The PCR was conducted in a final volume of 50 μL using the Isis™ Core Kit (MP Biomedicals LLC) according to the manufacturer's recommendations. The 2 kb PCR product was ligated into the cloning vector pQE30 (Qiagen, United Kingdom) in-frame with an N-terminal RGS-(His)6 tag to give pNZ50 which thus codes for a His-tagged rBteA protein. pNZ50 was verified for *bte*A insertion by DNA sequencing and was used to transform the *Escherichia coli* strain NEB Express I^q (New England Biolabs) according to the manufacturer's protocol. For protein production, pre-cultures starting from an isolated colony in LB medium supplemented with 100 μg/ml

ampicillin and 10 μg/ml chloramphenicol were incubated at 30 °C overnight. An aliquot of this culture was used to seed a larger volume of the same culture broth at an initial OD_{650} of 0.1. The bacteria were grown in the same conditions until the culture reached an OD_{650} of 0.5. Production of the rBteA was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and the culture incubated for a further 45 min.

2.3. Purification of recombinant His-6-BteA

All purification steps were conducted at 4 °C. IPTG-induced *Escherichia coli* cultures were chilled on ice and centrifuged at 8000 g for 20 min. The supernatant was discarded and the pellet stored at −20 °C until purification. The pellet was resuspended in 10 ml of 5 mM imidazole, 1 mg/mL lysozyme in buffer A (500 mM NaCl; 50 mM Tris−HCl; pH 8) and the bacterial suspension sonicated. The lysate was centrifuged at 8600 g for 30 min and the supernatant filtered through a 0.22 μm pore-size Millex filter (Millipore). The rBteA protein was purified from the corresponding lysate on the basis of its hexahistidine tag by affinity chromatography on a Ni-NTA resin (Qiagen, United Kingdom). The protein was eluted with a step gradient of imidazole (60, 100 and 200 mM). Resulting fractions were either kept in these conditions or dialyzed in order to get rid of imidazole which was replaced by Tris−EDTA 5 mM buffer in order to avoid any complex that might occur between the 6-His tag of the recombinant protein and the free cations that might be present in the solution after purification. Protein concentrations were determined by Bradford assay and these fractions were then analyzed by SDS-PAGE under reducing conditions with 10 μg proteins per well. The fractions containing rBteA were analyzed using RGS-His™ antibody (Qiagen, United Kingdom) recognizing the His6 tag on rBteA. The fraction containing rBteA was then stored in Tris−EDTA buffer at −20 °C.

Mass spectrometry analysis was also conducted on the purified fraction of the recombinant protein. After electrophoretic migration on an acrylamide gel, proteins were stained with Coomassie blue and analyzed with a 4800 Maldi-Tof/Tof device. Spectrograms were analyzed against the NCBInr bank in order to characterize the proteins present in the gel.

A mock control, consisting of a non-transformed NEB Express I^q *E. coli* strain was analyzed and processed under the same conditions.

2.4. Production of polyclonal serum against recombinant His6-BteA (rBteA)

To obtain specific sera against rBteA, 4 week-old Balb/c mice (Janvier, France) were immunized subcutaneously with 10 μg rBteA emulsified in Stimune Adjuvent (Prionics, Swiss) according to the manufacturer's protocol and boosted on days 21, 37 and 90 with the same preparation. Mice were bled 30 days after the last immunization and the serum was tested for the presence of specific antibodies (Abs) against rBteA by western blotting.

2.5. Western blot analyses

Bacteria from 24 h BGA cultures were suspended at a final OD_{650} of 6 in saline buffer and were diluted at a 1:1 ratio in Laemmli buffer (BioRad) according to the manufacturer's recommendations. Liquid SS cultures at a final OD_{650} of 1 were concentrated to an OD_{650} of 6 and were then processed as described above. The samples were run on Criterion™ TGX Stain-Free 4−15% acrylamide gels (BioRad) in denaturing conditions with a PageRuler™ Plus Prestained Protein Ladder (MW, Fermentas). The proteins were transferred to a nitrocellulose membrane (Protran, Whatmann) and stained with Ponceau Red in order to confirm the correct transfer after blotting. Membranes were then probed with polyclonal murine anti-rBteA antibodies at a 1:5000th dilution in 5% non fat milk in TBS 1× followed by incubation with ECL™ anti-mouse IgG (GE HealthCare) conjugated with a horseradish peroxidase at a 1:20000th dilution in the same buffer. SuperSignal™ West Dura (Thermo Scientific) was used for revelation according to the manufacturer's protocol.

For detection of specific antibodies in mouse anti-sera, purified rBteA (this study), PT (GlaxoSmithKline) and AC-Hly [21] were separated and transferred as described above. Membranes were then probed with different mouse serum at a final concentration of 1:250th followed by incubation and revelation with ECL™ anti-mouse IgG and SuperSignal™ West Dura as already described.

2.6. DNA extraction and genotyping

Total bacterial DNA was extracted with DNeasy Blood and Tissue Kits (Qiagen) according to the manufacturer's recommendations. The intergenic region between *btc*A and *bte*A was genotyped according to [12]; *bte*A was genotyped with the primers used for cloning plus an additional internal primer BP0500_751for (5′-CCGCAATGGCAGGAATACAC-3′). Genotyping of virulence factors was conducted as described [22,23].

2.7. Murine model of respiratory infection

All procedures involving animals were conducted in accordance with the Institut Pasteur animal care and use committee guidelines.

LD50 determinations were conducted as described [9].

Specific anti-BteA, AC-Hly, and PT antibodies in the serum of female Swiss mice intranasally infected at four-week of age with 1.10^6 bacteria were detected by western blotting as described in Ref. [24].

3. Results

3.1. Preparation of mouse polyclonal anti-rBteA antibodies

Protein purification was conducted on IPTG-induced pNZ50-transformed *E. coli* cultures as well as on a mock control consisting of untransformed Neb Express I^q *E. coli*

cultures. The different protein fractions were analyzed by SDS-PAGE and Coomassie Blue staining (Fig. 1A). While the imidazole eluted preparation consisted in a mixture of several proteins migrating at different apparent molecular weights (Fig. 1A lane 1), the Tris–EDTA fraction mainly contained a protein migrating with an apparent molecular weight of 78 kDa (Fig. 1A lane 2) whereas the mock control extract contained a protein migrating with an apparent molecular mass of 74 kDa (Fig. 1A lane 3).

Mass spectrometry analysis revealed that all these proteins were rBteA, in imidazole and Tris–EDTA buffer, confirming mono- and multi-meric forms of the recombinant protein in these conditions, as well as proteolytic forms. The 74 kDa protein found in the mock control preparation was found to be the glucosamine-6-phosphate from *E. coli*, co-purified with rBteA and also present in the induced purified extracts (Fig. 1A lane 1, 2 and 3). This protein exists in *Bordetella* species but only shares 54% homology with this 74 kDa *E. coli* protein.

Western blot analysis of these samples and total cell extracts with an anti-His antibody, confirmed that the total cell extract as well as the imidizole-purified preparations contained markable amounts of multi- and mono-meric forms of rBteA, whereas the Tris–EDTA fraction contained almost no multimeric forms of rBteA (Fig. 1B lanes I, 1 and 2). No leakage was found in the mock control (Fig. 2B lanes M and 3)

This recombinant protein was immunogenic and induced an anti-rBteA immune response in Balb/c mice (Fig. 1C, lane I, 1 and 2) identical to profiles obtained with the anti-His antibody. Even though present in little amounts, the 74 kDa *E. coli* protein did not produce a detectable response *in-vivo*. Indeed, it is not recognized by the polyclonal anti-rBtea anti-serum in the total cell extract or purified fraction of the mock control (Fig. 1C, lane M and 3).

3.2. In-vitro *production of BteA by* Bordetella *isolates*

The reference strains *B. pertussis* Tohama, *B. parapertussis* 12822, *B. bronchiseptica* RB50, the French wP vaccine strains Bp1414 and Bp1416 and the *Bordetella* clinical isolates were screened for BteA production.

Both *B. pertussis* and *B. bronchiseptica* reference strains as well as the French wP vaccine strains Bp1414 and Bp1416 produce BteA, whereas the *Bordetella parapertussis* reference strain 12822 does not (Fig. 2A).

BteA was detected in both *B. pertussis* Tohama and *B. bronchiseptica* RB50 cultured in modified Stainer Scholte medium or on BGA culture plates (data not shown). Consequently, all isolates were screened for BteA production after culture on BGA culture plates.

BteA was detected in 99.0% (293/296) of tested *B. pertussis* isolates whereas 0.0% (0/14) of tested *B. parapertussis* isolates produce this toxin *in-vitro*. 97.5% (79/81) of tested *B. bronchiseptica* isolates produced BteA (Table 1 and Fig. 2B).

Migration profiles show that BteA produced by *B. pertussis* isolates migrates to a lower apparent molecular weight compared to BteA produced by *B. bronchiseptica* isolates.

Subculturing BteA+ isolates five times did not lead to the loss of BteA production, unlike what has been described for Bsp22 [25]; neither did subculturing lead to BteA production in BteA- isolates (data not shown).

Secretion experiments were conducted in Stainer Scholte synthetic liquid medium on selected *B. pertussis* and *B. bronchiseptica* reference strains and isolates. The *B. pertussis* Tohama reference strain actively secreted PT or AC-Hly in the culture supernatant (data not shown), demonstrating that the strain was indeed secreting virulence factors. Nevertheless, no BteA was found at any tested time point in the culture supernatant whereas this toxin was found in the bacterial pellet (Fig. 2C) confirming that it was produced by *B. pertussis* Tohama grown in these conditions but mainly remaining in the cytosolic compartment. On the contrary, BteA was found in the culture supernatant of RB50 (Fig. 2C), the *B. bronchiseptica* reference strain. The absence of PRN in the supernatant confirmed that BteA was actively secreted and not released after cell lysis (data not shown).

No trace of BteA could be found in any other tested *B. pertussis* isolate's supernatant (0/4) whereas it was retrieved

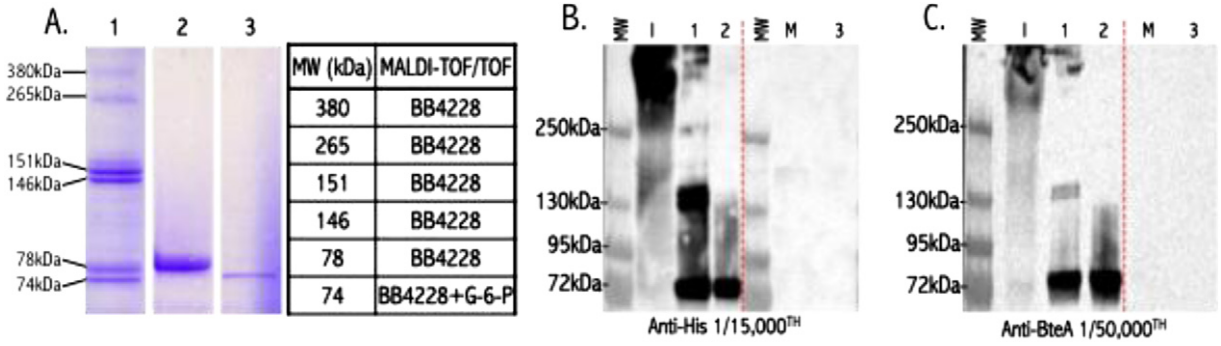

Fig. 1. *Purification of rBteA and polyclonal mouse anti-rBteA testing.* (A) Coomassie blue staining of the imidazole eluted fraction from Ni-NTA columns (1) and the Tris–EDTA dialyzed fraction (2) of IPTG-induced *E. coli* pNZ50 cultures (expected monomer molecular weight of 69 kDa) as well as the Ni-NTA purified fraction of non-transformed NEB express I^q *E. coli* cultures (3). These samples, in addition to a total cell extract of IPTG-induced pNZ50-transformed *E. coli* cultures (I) and a total cell extract of untransformed NEB express I^q *E. coli* cultures (M) were transferred to a nitrocellulose membrane and probed with (B) anti-RGS-His antibodies or (C) polyclonal murine anti-rBteA anti-sera obtained after immunization of Balb/c mice with the rBteA preparation.

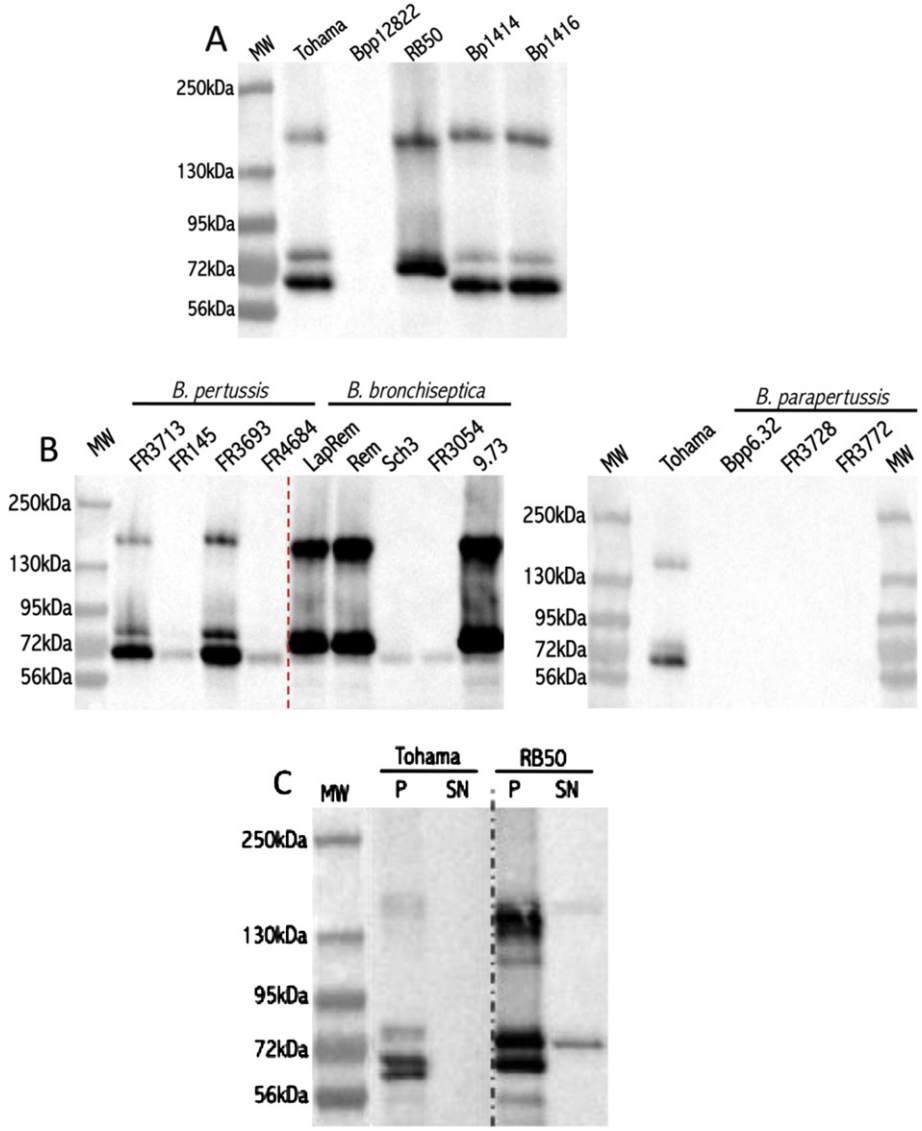

Fig. 2. *Detection of BteA production in bacterial suspensions of Bordetella isolates using western blot analysis.* Detection of BteA production in bacterial suspensions of (A) *B. pertussis* Tohama, *B. parapertussis* 12822, *B. bronchiseptica* RB50 and *B. pertussis* French wP vaccine strains Bp1414 and Bp1416 cultured on BGA medium and of (B) selected *Bordetella* isolates cultured on solid BGA medium. (C) Detection of BteA in pellets (P) and culture supernatants (SN) of *B. pertussis* Tohama and *B. bronchiseptica* RB50 reference strains grown in Stainer Scholte liquid synthetic medium.

from the bacterial pellets. These isolates were also secreting PT and AC-Hly. Only one *B. bronchiseptica* isolate (Rem) out of eight tested was found not to secrete BteA whereas secreting AC-Hly. The seven remaining isolates were able to actively secret BteA unrelated to cell lysis.

3.3. Genotyping the promoter region of bteA

The *bteA* promoter regions of the three *B. pertussis* BteA- isolates carry a 481 like insertion sequence (IS481 like) as described by Han et al. [12], explaining the BteA- phenotype.

10 pre-vaccine, 18 post-wP vaccine and 19 post-aP vaccine *B. pertussis* isolates were tested for their *bteA* promoter sequence. This region was highly conserved among these 47 *B. pertussis* isolates: there was only one single nucleotide polymorphism (A to G at position −207 bp from the *bte*A translation start codon) as described [12,16].

The *bte*A promoter region in each of the fourteen *B. parapertussis* human isolates was 100% identical to that in the reference strain 12822.

Genotyping the *bte*A promoter region of 15 *B. bronchiseptica* isolates of human origin and 11 *B. bronchiseptica* isolates of animal origin revealed a strong conservation of this region. 72.0% (18/25) of the tested isolates presented a 100% identity with the sequence of the reference strain RB50, irrespective of their human (11/18) or animal (7/18) origin. Sequencing this region nevertheless showed a higher diversity among the *B. bronchiseptica* population compared to *B. pertussis* or *B. parapertussis*. No mutation was found in the *bteA* promoter that could explain the BteA- phenotype of one

Table 1
Vaccine era of collection of *B. pertussis* and *B. parapertussis* isolates and origin of collection of *B. bronchiseptica* isolates screened for production of BteA.

Species	Era/Origin	Tested/BteA-	Total/BteA−[a]	% BteA+[b]
B. pertussis	pre-vaccine	14/0	296/3	99.0%
	post-wP	68/1		
	post-aP	214/2		
B. parapertussis	pre-vaccine	2/2	14/14	00.0%
	post-wP	2/2		
	post-aP	10/10		
B. bronchiseptica	Human	31/3	81/3	96.3%
	Animal	50/0		

[a] Total number of isolates screened for BteA production for each species/number of isolates not producing BteA for each species.
[b] Proportion of isolates producing BteA among all tested isolates.

B. bronchiseptica isolate (Sch, Table 2), as its promoter is 100% identical to that in RB50. The promoter region of the second BteA- *B. bronchiseptica* isolate (FR3054, Table 2), shares 99% identity with the promoter region of the *B. parapertussis* reference strain 12822, but only 92% (19 different bases out of 248 bases compared) with that of *B. bronchiseptica* RB50. The *bteA* gene harbored by this isolate (EMBL accession number HE974463) shares 99% identity with the *B. parapertussis* reference strain 12822 and only 97% identity (52 different bases out of 1977 compared) with the *B. bronchiseptica* reference strain RB50.

Table 2
Characteristics of *B. pertussis* and *B. parapertussis* *B. bronchiseptica* isolates tested in the murine model of respiratory infection.

B. pertussis	Name	Era	BteA[a]	In-vivo[b]	LD50	Ref
	Tohama	pre-vaccine	+	−	8.10^7	[9]
	18323	pre-vaccine	−	−	2.10^6	[24]
	FR145	post-wP	−	nt[c]	5.10^7	[10]
	FR3693	post-aP	+	−	6.10^7	[9]
	FR3713	post-aP	+	−	2.10^7	[10]
	FR4684	post-aP	−	nt	1.10^7	this study
B. parapertussis	Name	Era	BteA	In-vivo	LD50	Ref
	63.2	pre-vaccine	−	nt	8.10^7	[10]
	12822	post-wP	−	−	1.10^8	[46]
	FR3743	post-aP	−	nt	3.10^7	[10]
	FR3772	post-aP	−	nt	6.10^7	this study
B. bronchiseptica	Name	Origin	BteA	In-vivo	LD50	Ref
	RB50	rabbit	+	nt	3.10^6	[33]
	Del	human	+	+	5.10^3	[47]
	9.73	rabbit	+	+	3.10^6	[47]
	LapRem	rabbit	+	−	2.10^6	[47]
	Jay	human	+	+	2.10^7	this study
	Por	pig	+	+	5.10^5	this study
	Rem	human	+	−	1.10^8	[47]
	Sch	human	−	nt	NL[d]	this study
	FR3054	human	−	nt	NL	this study
	FR3670	human	+	+	8.10^5	this study

[a] Production of BteA tested by Western blot analysis. "+" indicates that the isolate produces BteA *in-vitro* when cultured on BGA agar plates. "−" indicates that BteA is not produced in these conditions.
[b] Sera collected from infected mice 90 days after infection were tested for an anti-BteA immune response. "+" indicates that the serum contained antibodies directed against BteA, "−" indicates that such antibodies were absent.
[c] nt: not tested.
[d] NL: not lethal in the mouse respiratory model. LD50 values are reported as CFU/mouse.

3.4. Induction of anti-BteA immune response in-vivo

The *B. pertussis* reference strain Tohama and two clinical isolates, FR3713 and FR3693, were tested for their ability to induce an anti-BteA immune response *in-vivo* using the murine model of respiratory infection (Fig. 3A). Although producing BteA *in-vitro*, the reference strain and the two isolates collected during the post-aP vaccine era did not induce an anti-BteA immune response *in-vivo*. Nevertheless, these bacteria induced an immune response against the other virulence factors, such as AC-Hly and PT (Fig. 3A), indicating that mice were correctly infected and that *B. pertussis* induced an *in-vivo* humoral immune response during infection of the mice. As for *B. pertussis*, the *B. parapertussis* reference strain 12822 was unable to induce an anti-BteA immune response (data not shown).

Four *B. bronchiseptica* isolates collected from humans (Del, Jay, Rem and FR3054), two isolates collected from rabbits (9.73 and LapRem) and one isolate collected from a pig (Por) were also tested for their ability to induce an anti-BteA immune response *in-vivo*. All these isolates produce BteA *in-vitro*. LapRem and Rem, two isolates associated with a persistent case of *B. bronchisepetica* infection [26], did not induce an anti-BteA immune response, but did induce an immune response directed against AC-Hly (Fig.3B). The five other isolates all induced the production of anti-BteA as well as anti-AC-Hly antibodies (not shown) in the murine model after a respiratory infection (Fig. 3C).

3.5. LD50 of isolates producing or not producing BteA

All tested *B. pertussis* isolates were lethal in the murine model of respiratory infection provided they produced PT and/or AC-Hly. There was no difference in the LD50 values whether or not these isolates produced BteA *in-vitro* (Table 2). Although being all BteA-, all tested *B. parapertussis* isolates were lethal in the same model (Table 2).

B. bronchiseptica isolates that produced BteA *in-vitro* were lethal in the murine model of respiratory infection whereas BteA- isolate FR3054 producing AC-Hly, FHA and PRN was not lethal. Similarly, the second BteA- *B. bronchiseptica* isolate Sch, which also does not produce AC-Hly was not lethal neither (Table 2).

4. Discussion

To colonize the respiratory tract and infect their hosts, *Bordetella* species display an array of virulence factors. Whereas adhesins act as anchors to enable the adherence and colonization, toxins act to subvert the immune response and in turn prevent the rapid clearance of the bacteria. Collectively these virulence factors enable the establishment of the bacterial infection in the host respiratory tract. *Bordetella* virulence factors include toxins of high importance as knock down or

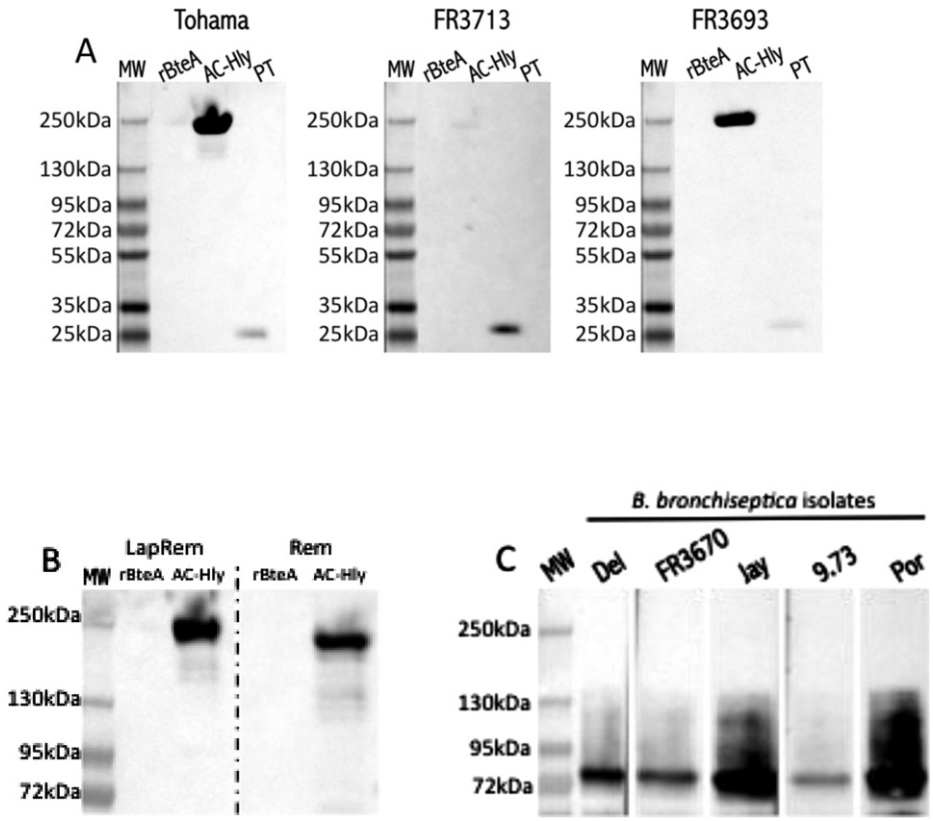

Fig. 3. *Detection of the immune response of mice infected with Bordetella isolates using western blot.* Immune response directed against (A) rBteA, AC-Hly and PT by polyclonal mice anti-sera obtained after mice infection with *B. pertussis* reference strain Tohama or two post-aP clinical isolates FR3713 or FR3693, (B) rBteA and AC-Hly by polyclonal mice anti-sera obtained after mice infection with *B. bronchiseptica* LapRem or Rem and (C) rBteA by polyclonal mice anti-sera obtained after mice infection with *B. bronchiseptica* 9.73 or Del or FR3670 or Jay or Por. Sera used for this experiment were sampled at day 90 post-infection.

abolition of their production can lead to a total loss of the bacterial capability to colonize the respiratory tract in a murine model of respiratory infection [9,27–29]. *B. bronchiseptica* has been suggested to be the ancestral progenitor of *B. pertussis* and *B. parapertussis* which may have emerged from distinct lineages. These three species are thus closely related [3,4] at the genetic and proteomic level. But, unlike *B. pertussis* and *B. parapertussis*, *B. bronchiseptica* has retained its ability to invade a wide range of four legged mammals and also, in some particular conditions, even humans [26].

Genome-wide screening of *B. bronchiseptica* reference strain RB50 led to the discovery of *bte*A, encoding the potent cytotoxic effector, BteA [13]. This toxin induces non-apoptotic cell death in a wide range of mammalian cells. The *bte*A gene is highly conserved and functionally interchangeable [30] among *B. bronchiseptica*, *B. pertussis* and *B. parapertussis*, with 96%–99% homology between these species, and encodes a peptide of a predicted molecular mass of approximately 68 kDa (658, 656 and 657 residues in these three species, respectively).

For toxins to act during infection, they must be produced and attain their target. BteA exerts its cytotoxic activity at the cytoplasmic face of target cell membranes [30] in a TTSS dependant manner.

TTSS is a well-known intracellular delivery device for Gram negative bacteria to inject effector proteins directly into the cytosol of target cells through a needle-like secretion system. Amongst the *Bordetella* genus it was first described in *B. bronchiseptica* [31] and was shown to play an important role during infection in mouse models of respiratory infection for *B. bronchiseptica* and *B. pertussis* [15,32–34]. In *Bordetella* species, the TTSS is encoded by the *bsc* locus, which is highly conserved between *B. bronchiseptica*, *B. pertussis* and *B. parapertussis* [35]. Several studies suggest that the *bsc* locus is transcribed, regulated and expressed in *B. bronchiseptica* and *B. pertussis* whereas its expression in *B. parapertussis* has been suggested but not yet demonstrated [12,14,15,36]. TTSS function relies on the correct expression of numerous proteins, each of which is essential for building the injectisome and for the translocation of TTSS substrate proteins. The lack or mis-expression of key factors abolishes TTSS substrate secretion and in turn TTSS function [35]. The transcription of the *bsc* locus, as for *bteA*, is under the direct control of the Bordetella TTS regulator (*btr*) *btr*S/*brpL* (Gene ID BP2234, BPP2241, BB1638) [13,14], which shares similarities with ECF Sigma Factors and is classified as an RNA polymerase sigma factor. All three species are thus expected to produce BteA.

4.1. In-vitro *production of BteA by Bordetella species*

BteA has been mainly described in *B. bronchiseptica* [13,16,30] and more recently in *B. pertussis* [12]. We

demonstrate that BteA is produced in almost all the French *B. pertussis* (293/296) and *B. bronchiseptica* (79/81) isolates analyzed, whatever their era or origin of collection. By contrast, the 14 human *B. parapertussis* isolates tested did not produce it. Nevertheless, although produced, BteA remains cytosolic for *B. pertussis* isolates tested in our conditions whereas *B. bronchiseptica* isolates are capable of secreting this toxin in the culture supernatant.

Unlike its ancestor *B. bronchiseptica*, *B. pertussis* is a strictly human pathogen; the development and use of wP vaccines in the late 1950s therefore imposed a new selective pressure on this species, leading to a change in *B. pertussis* population circulating in vaccinated populations [6,17,37–39]. Introduction of aP vaccines and increased vaccine coverage in France established a new challenge for *B. pertussis* isolates still circulating. Loss of PRN production seems to be the most frequent response of *B. pertussis* isolates to aP vaccine-induced immunity [8,10,11]. We observed no particular differences in BteA production between isolates from different eras of collection unlike what was reported by Han and co-workers [12]: 99.0% of all *B. pertussis* isolates tested produce BteA including the French vaccine strains or the Tohama reference strain, also used for Japanese vaccine production. The three isolates that do not produce this toxin carry an IS481-like element in the *bte*A promoter region and harbor a *prn1* allele and could thus be considered vaccine-type isolates, as already described [12]. But unlike Japanese isolates, these isolates were not prevalent before wP vaccine introduction; we found no evidence that vaccine-induced immunity favored the emergence of BteA+ isolates, unlike what has been suggested by Han and co-workers [12] for Japan.

All tested *B. parapertussis* isolates were BteA-, and the reason for this phenotype remains unknown. The promoter region in this species shares only 92% identity with those in *B. bronchiseptica* and *B. pertussis* and might impact *bteA* transcription and BteA production. Another possible explanation might reside in BtrS, the master regulator belonging to the *btr* locus [14]; *btrS* is considered a pseudo-gene and does not have any stop codon in *B. parapertussis* reference strain 12822; it is thus the most plausible explanation so far for the BteA- phenotype of the *B. parapertussis* isolates. It is nevertheless intriguing that *B. parapertussis* was formerly suggested to express *btr* genes as well as TTSS substrates *bsp22* and *bopN* [14]. Transcriptomic analysis on *bteA* and *btr* genes are currently undertaken to verify these hypothesis. Nevertheless, although *B. parapertussis* seems to be affected by aP vaccine-induced immunity [10,40], wP or aP vaccines did not foster the emergence of BteA+ *B. parapertussis* isolates as no production of this toxin was found in any isolates whatever it's era of collection. In addition, the promoter region of the *bte*A gene and the gene itself do not present any polymorphism, indicating that there has been no genetic evolution in this region since vaccine introduction; this contrasts with *B. pertussis* isolates for which we also report a single nucleotide polymorphism possibly linked to selection pressure from the wP vaccine [12,25,41].

Only two *B. bronchiseptica* isolates did not produce BteA and although isolated from humans, no link could be established between the isolates' origins and phenotypes. The promoter region of *bte*A and *bte*A itself in *B. bronchiseptica* Sch isolate did not differ from those in the reference strain RB50. Preliminary transcriptomic analyses show that *btrS* is less transcribed in this isolate compared to the reference strain RB50, leading to a decrease in *bteA* transcription (in preparation). TTSS regulation is complex, and a BteA- phenotype can result from disruption elsewhere in the system. This isolate produces other *bvg*-regulated virulence factors, excluding the involvement of BvgAS in the BteA- phenotype, which is confirmed with our preliminary transcriptomic analysis; the involvement of *btr*S is currently under investigation. On the contrary, the promoter region of the *bte*A gene in FR3054, the second BteA- *B. bronchiseptica* isolate, is very divergent from that in the reference strain RB50; it is more similar to the promoter region found in *B. parapertussis* isolates that do not produce BteA. Investigation of possible signal sequence divergence in the promoter region did not provide any evidence of the involvement of this region in the BteA- phenotype. Currently, the most plausible explanations for this phenotype involves *btr*S (under investigation).

4.2. In-vivo *production of BteA by* Bordetella *species and lethality of* Bordetella *isolates in the murine model*

Although producing BteA *in-vitro* ([12] and this study), *B. pertussis* isolates fail to induce an anti-BteA immune response in a mouse model of respiratory infection. Similarly, *B. pertussis* is not able to induce Human respiratory epithelial cell (12 and Guillemot, Njamkepo, Guiso, *Bordetella* meeting, 2006, Paris) or HeLa cell necrosis *in-vitro* [14]. This phenotype might be linked to the incapacity of *B. pertussis* to effectively secrete BteA into the extracellular compartment, phenotype probably related to a TTSS defect. Nevertheless, the LD50 in the murine respiratory model is similar for *B. pertussis* isolates producing BteA *in-vitro* or not ([10] and this study). These data suggest that BteA production *in-vivo* is not mandatory for *B. pertussis'* virulence, as BteA- isolates still producing the major toxins and adhesins as PT, AC-Hly, FHA, PRN and FIM [10] remain as lethal as BteA+ isolates. However, these observations are from mouse and *in-vitro* models, and *B. pertussis* is a strictly human pathogen. Possibly, the effects of *B. pertussis* BteA *in-vivo* may be dependent on a more complex process or specific signaling. Indeed, this toxin is still produced and secreted by *B. pertussis* isolates ([12] and this study) despite appearing to be inessential for virulence in *in-vivo* and *in-vitro* laboratory models.

No anti-BteA antibodies were detected in the serum of mice infected with human *B. parapertussis* isolates. This is not surprising, as these isolates do not produce BteA *in-vitro*. Nevertheless, it demonstrates that, at least in the murine model of respiratory infection, no specific signaling induces BteA production in *B. parapertussis* isolates.

The *B. bronchiseptica* isolates DEL, JAY and FR3670 of human origin, 9.73 of rabbit origin, and POR of pig origin, all produce BteA *in-vitro* and were able to induce anti-BteA antibodies *in-vivo*, indicating the *in-vivo* production of this toxin.

Isolate Rem of human origin, and LapRem of rabbit origin, both *in-vitro*-BteA+ *B. bronchiseptica* isolates, failed to induce such antibodies. Whereas the lack of BteA secretion by the *B. bronchiseptica* Rem isolate might explain such a phenotype, it appears that the *in-vivo* transcription of *bteA* and production of BteA answers to specific signaling which remain to be discovered. However, all seven isolates are lethal in the murine respiratory model of infection [26,42], whereas FR3054 (producing AC-Hly) and Sch (not producing AC-Hly), the BteA- *B. bronchiseptica* isolates, are not.

5. Conclusion

B. pertussis and *B. parapertussis* may have emerged from *B. bronchiseptica* through spontaneous genomic rearrangements [3]. Through gain or loss of protein production, ancestral species acquired selective advantage and managed to colonize new hosts, thereby avoiding competition and evolving independently towards these two new species.

Vaccination strategies have affected *B. pertussis* and possibly *B. parapertussis* population evolution over recent decades but has not impacted BteA production in these species in France unlike what has been suggested in Japan by Han and co-workers [12]. It nevertheless appears that the only BteA- *B. pertussis* French isolates can be considered as vaccine-type isolates as described [12]. One possible explanation for the divergence between Japan and France is the difference in vaccination strategies and the fact that wP vaccine has been used for more than thirty years in France, unlike in Japan, controlling the population of vaccine-type isolates [10,17,37] that possibly still circulate in higher proportion in Japan.

B. pertussis isolates seem to only produce BteA *in-vitro* and *B. parapertussis* isolates do not produce BteA at all. Nevertheless these two species still circulate in the population. PT and AC-Hly for *B. pertussis*, LPS for *B. parapertussis* play crucial roles during infection [2] and it appears here that BteA might play a crucial role for *B. bronchiseptica* during infection although other factors might as well be involved and need to be verified in our isolates [43]. This toxin might have lost its lead during evolution for human adapted species but the universal production of BteA by *B. pertussis* isolates is puzzling. French wP vaccine strains produce BteA *in-vitro*, as well as the Japanese vaccine strain Tohama, and thus this toxin must have been part of wP vaccines for over thirty years in France. Nevertheless no genetic shift in *bteA* can be observed between vaccine-type or non-vaccine-type isolates as it was the case for *prn*, the promoter of *ptx* or *ptx* itself [5,6,38]. This toxin might either be very important for *B. pertussis* and thus is under a selective pressure as it is the case for AC-Hly [44], or it might also just be a vestige of a specialized function in an evolving organism. Considering our results regarding *B. pertussis* and *B. parapertussis*, the second hypothesis seems more likely to be correct unless BteA plays multiple roles and has activities not yet identified as for FHA [45]. This raises the question whether or not BteA must be considered a virulence factor in *B. pertussis*, rendering it a poor path to follow for future vaccination strategies.

Acknowledgments

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