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Genetic stability of the live attenuated *Bordetella pertussis* vaccine candidate BPZE1

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ABSTRACT

Despite the extensive use of efficacious pertussis vaccines, *Bordetella pertussis* infections are still among the main causes for childhood morbidity and mortality. Severe pertussis occurs mostly in very young children, often too young to be sufficiently protected by current vaccines, which require several administrations in regimens that vary between countries. Since natural infection with *B. pertussis* is able to induce protection, we have developed the live attenuated *B. pertussis* vaccine strain BPZE1 that protects mice upon a single intranasal administration. This strain was obtained by genetically inactivating pertussis toxin via two point mutations in the *ptx* gene, by deleting *dnt* encoding dermonecrotic toxin, and by replacing the *B. pertussis ampG* gene by *Escherichia coli ampG*, resulting in the removal of tracheal cytotoxin. Here, we assessed the genetic stability of BPZE1 after 20 and 27 weeks of continuous passaging in vitro and in vivo, respectively. BPZE1 was passaged 20 times in vitro and 9 times in vivo in Balb/C mice. After these passages, 8 hemolytic colonies were analyzed by PCR for the absence of *dnt* and *B. pertussis ampG* and the presence of *E. coli ampG*, by DNA sequencing for the presence of the two *ptx* point mutations and by DNA microarrays for the global genomic stability. In addition, the protective capacity of BPZE1 was evaluated after the passages. No genetic or protective difference was detected between the passaged bacteria and non-passaged BPZE1, indicating that stability of the vaccine strain is not a concern for BPZE1 to be considered as an attenuated live vaccine against whooping cough.

1. Introduction

Live attenuated vaccines offer a wide set of advantages over inactivated or acellular vaccines, not the least of which is the fact that they may be protective after a single administration and can often be administered by mucosal routes. It is the extensive use of a live attenuated vaccine, the small pox vaccine, that has led to the first eradication of a human disease. Most live attenuated vaccines in use have resulted from random mutagenesis, generally induced by serial passages of their virulent ancestors, but contain no deliberately designed genetic alterations. They are therefore at potential risk of reversion to virulence [1,2]. With the current knowledge of the molecular mechanisms of microbial virulence and the development of suitable genetic engineering tools, it is now possible to introduce precise deletions or mutations into virulent microorganisms, resulting in a rational attenuation with the potential to solve the problem of reversion to virulence of attenuated vaccines [3–6].

Engineering genetic attenuation mainly focuses on two principal groups of genes, those involved in a key metabolic pathway of the microbe and those involved in virulence and/or in the regulation of virulence genes [3,6,7]. We have developed BPZE1 [6], an attenuated *Bordetella pertussis* vaccine candidate, by basing the attenuation strategy on the knowledge of the molecular mechanism of *B. pertussis* virulence. The pathogenesis of whooping cough largely depends on the production of adhesins, important for colonization, and toxins [8,9]. BPZE1 is a derivative of virulent *B. pertussis* BPSM with the following three genetic alterations. The *dnt* gene encoding the dermonecrotic toxin was deleted, the *B. pertussis ampG* gene was replaced by the *Escherichia coli ampG* gene, thereby removing the tracheal cytotoxin, and the wild-type *ptx* gene coding for pertussis toxin was replaced by a mutated version coding

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for a genetically inactivated toxin derivative with R9K and E129G substitutions in the S1 subunit. These alterations yielded a highly attenuated strain that was still able to transiently colonize the mouse respiratory tract and to induce strong protective immunity against *B. pertussis* infection [6]. A single intranasal administration of BPZE1 induced full protection in adult mice (8 weeks old), as well as in infant mice (3 weeks old), whereas commercial acellular pertussis vaccine (aPV) only partially protected infant mice, even after repetitive doses. BPZE1 is therefore an attractive vaccine candidate to be administered very early in life, in order to protect infants, too young to be protected by the currently available vaccines. This is particularly relevant, since in spite of the extensive use of whole-cell vaccines and aPV, annually roughly 40,000,000 new cases of whooping cough and 300,000 pertussis-linked deaths are still reported today, mainly in infants younger than 6 months of age [10].

However, the use of genetically engineered live attenuated vaccines requires the vaccine strain to be stable, without risk of reversion to virulence. In this study, we investigated the genetic stability of BPZE1, in particular the stability of the introduced genetic alterations, over time through a series of in vitro and in vivo passages.

2. Materials and methods

2.1. Mice

Three-weeks-old (infant, purchased from Charles River, Arbresle, France) or eight-weeks-old (adult; Charles River) Balb/C female mice were maintained under specific pathogen-free conditions in the animal facilities of the Institut Pasteur de Lille, and all experiments were carried out under the guidelines of the Institut Pasteur de Lille animal study board.

2.2. Bacterial strains and growth conditions

B. pertussis BPSM [11] and BPZE1 [6] have been previously described. They were kept frozen at −80 °C suspended in phosphate-buffered-saline (PBS) in the presence of 30% glycerol. After thawing, they were grown at 37 °C on Bordet–Gengou (BG) agar (Difco, Laboratories) supplemented with 1% glycerol, 20% defibrinated sheep blood, and 100 μg/ml streptomycin.

2.3. Animal immunization and infection

Mice were slightly sedated with pentobarbital (CEVA Santé Animale - La Ballastière, France) and infected by pipetting 20 μl PBS containing approximately 10^6 colony-forming units (CFU) of *B. pertussis* BPSM or BPZE1 onto the tip of the nares. Groups of six animals were sacrificed at the indicated time points after inoculation, lungs were harvested, homogenized in PBS and plated in serial dilutions onto BG agar supplemented with 1% glycerol, 20% defibrinated sheep blood, and 100 μg/ml streptomycin. CFUs were counted after incubation at 37 °C for three days.

2.4. PCR analysis of the ampG and dnt loci of BPZE1

Genomic DNA (gDNA) was extracted from selected isolated colonies of *B. pertussis* BPZE1 on BG agar plates or from pooled bacteria after the final passages, using the genomic DNA extraction kit (QIAGEN). The individual colonies were suspended in 100 μl H_2O, heated for 20 min at 95 °C and centrifuged for 5 min at 15,000 × *g*. One microlitre of supernatant was then used as template for the PCR, using the sense (S) and anti-sense (A) oligonucleotides described in Table 1. The PCRs were carried out for 30 cycles, and the amplified products were analyzed by electrophoresis within a 1% agarose gel in TAE buffer containing ethidium bromide and visualized under UV light.

Table 1
List of primers used in this study

Genes	Product length (bp)	Primer sequence[a] (5′–3′) and (3′–5′)
E. coli ampG[b]	402	S: ATGTGCTTCCGGCAGAAGAA A: CAAGCGTTTTGTTAACCACG
B. pertussis ampG[b]	659	S: TCGCAGGACATCGCCTTCGA A: ATCAGCAGCGCCACGAAGGA
dnt flanking regions[b]	1511	S: ATACCATGGCGCCGCTGCTGGTGCTGGGC A: ATATCTAGACGCTGGCCGTAACCTTAGCA
dnt internal region[b]	882	S: TATAGAATTCGCTCGGTTCGCTGGTCAAGG A: ATCCTGGCCGATGGGTTCAT
ptx[b]	620	S: AGACGGGATGCGTTGCACTC A: TCTGCTGGCTGACGTAGCGA
SeqR9k[c]		CCAAACCGCAAGAACAGGCT
SeqE129G[c]		AATACTCCGTGGTCGTGGTCTCGC

[a] Sequences correspond to *E. coli ampG* (accession number, X82158), *B. pertussis ampG*, *dnt*, *ptx* (accession number, BX470248).
[b] Primers used for the amplification of indicated DNA fragments.
[c] Primers used for sequencing of the amplified *ptx* DNA fragment.

2.5. Sequence analysis of the ptx locus of BPZE1

The DNA fragments containing the region encompassing the R9K and the E129G mutations of the *ptxS1* gene were amplified by PCR from bacterial genomic DNA isolated from selected colonies as described above, using primers shown in Table 1. The amplified DNA fragments were then directly sequenced in both directions by automated sequencing.

2.6. DNA microarray analyses

The *B. pertussis* DNA microarray was designed as described previously [12–14]. The microarray was based on PCR products of nearly all *B. pertussis* BPSM open reading frames spotted onto aldehyde-activated glass slides (Nexterion Slide AL, Schott Nexterion) by using the Q-Array II spotter (Genetix) in a 54% relative humidity and at 20 °C. The spotted slides were then incubated in a humidity chamber (>90% relative humidity) at room temperature for 15 min and finally at 120 °C for 60 min. Before use, the slides were washed twice in 0.2% SDS at room temperature, rinsed twice in distilled water at room temperature, denatured for 3 min in water at 95 °C and rinsed again once in distilled water. The slides were then incubated at room temperature in 10.6 mM $NaBH_4$, 0.75 × PBS, 25% absolute ethanol prepared 30 min before use, rinsed twice in 0.2% SDS for 2 min each and twice in distilled water at room temperature. Finally, they were dried by centrifugation at 500 rpm for 5 min at room temperature.

Twenty microgram of *B. pertussis* gDNA was fragmented by digestion in 20 units of *Mbo*I (5 U/μl, New England Biolabs) at 37 °C for 2 h. Fragmentation was verified by electrophoresis in a 1.5% agarose gel. The fragmented gDNA was then purified using the phenol–chloroform–isoamyl alcohol (25:24:1) extraction followed by ethanol precipitation. Two microgram of fragmented gDNA was labeled by nick translation using the Amersham Nick Translation Kit (GE Healthcare) in the presence of 30.4 μM of each dGTP, dATP, dTTP, and 1 mM of Cy3-labeled dCTP or Cy5-labeled dCTP (PerkinElmer). After incubation in the dark at 15 °C for 5 h, the labeled fragmented gDNA was purified by using the Qiaquick PCR purification Kit (Qiagen). The