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Construction and evaluation of *Bordetella pertussis* live attenuated vaccine strain BPZE1 producing Fim3

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ABSTRACT

Pertussis or whooping cough is currently the most prevalent vaccine-preventable childhood disease despite >85% global vaccination coverage. In recent years incidence has greatly increased in several high-income countries that have switched from the first-generation, whole-cell vaccine to the newer acellular vaccines, calling for improved vaccination strategies with better vaccines. We have developed a live attenuated pertussis vaccine candidate, called BPZE1, which is currently in clinical development. Unlike other pertussis vaccines, BPZE1 has been shown to provide strong protection against infection by the causative agent of pertussis, *Bordetella pertussis*, in non-human primates. BPZE1 is a derivative of the *B. pertussis* strain Tohama I, which produces serotype 2 (Fim2) but not serotype 3 fimbriae (Fim3). As immune responses to fimbriae are likely to contribute to protection, we constructed a BPZE1 derivative, called BPZE1f3, that produces both serotypes of fimbriae. Whereas nasal vaccination of mice with BPZE1 induced antibodies to Fim2 but not to Fim3, vaccination with BPZE1f3 elicited antibodies to both Fim2 and Fim3 at approximately the same level. In mice, both BPZE1 and BPZE1f3 provided equal levels of protection against clinical isolates that either produce Fim2 alone, both Fim2 and Fim3, or no fimbriae. However, vaccination with BPZE1f3 provided significantly stronger protection against Fim3-only producing *B. pertussis* than vaccination with BPZE1, indicating that immune responses to fimbriae contribute to serotype-specific protection against *B. pertussis* infection.

1. Introduction

Whooping cough or pertussis is a severe respiratory disease that can be life-threatening, especially in young infants, and its incidence is on the rise in several countries, despite a global vaccination coverage of >85% [1]. However, it also affects adolescents and adults, where symptoms are usually atypical, and therefore the disease often remains undiagnosed in these age groups [2]. Nevertheless, adolescents and adults, even if they remain asymptomatic, can transmit the causative agent *Bordetella pertussis* to young infants before they are protected by the primary vaccination series. In fact, a recent wavelet analysis of *B. pertussis* infection in the US and the UK, combined with a phylodynamic analysis of clinical isolates suggested that asymptomatic transmission is the principal cause of the recent pertussis resurgence [3]. In addition, asymptomatic *B. pertussis* infection may not be benign, as shown by recent reports, providing epidemiological evidence that *B. pertussis* infection may be related to auto-immune diseases, such as Celiac disease [4], multiple sclerosis [5], and even Alzheimer's disease [6].

Currently available whole-cell or acellular vaccines have been very effective in reducing the incidence of whooping cough after three primary vaccination doses [7]. However, in contrast to prior infection with *B. pertussis*, they are much less effective in reducing asymptomatic colonization, as shown in the recently established baboon model [8]. Although vaccinated baboons were protected

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against pertussis disease following experimental infection with *B. pertussis*, they could readily be infected and transmit the organism to co-housed animals, even in the absence of symptoms, in contrast to convalescent baboons. Altogether these observations illustrate the shortcomings of currently available vaccines, and call for new vaccines that protect against both disease and infection.

Based on the observation that the best way to protect against *B. pertussis* colonization is prior infection, we have developed a live attenuated vaccine that can be administered by the nasal route, in order to mimic as much as possible natural infection without causing disease. The vaccine strain, called BPZE1, lacks the gene coding for dermonecrotic toxin, produces genetically detoxified pertussis toxin and is deficient in tracheal cytotoxin production by the replacement of the *B. pertussis ampG* gene by *Escherichia coli ampG* [9]. BPZE1 has been shown to be safe in pre-clinical models, including in severely immunocompromized mice [10]. Although *B. pertussis* infection has been associated with autoimmune diseases and Alzheimer's disease [4–6], it is unlikely that BPZE1 will cause such diseases, as they are triggered by active PT [11], which is genetically inactivated in BPZE1. BPZE1 has also been found to be genetically stable after serial passages in vitro and in vivo for at least 12 months [12]. It protects mice against *B. pertussis* challenge after a single nasal administration [9,13], both via protective $CD4^+$ T cells and antibodies [14]. Furthermore, protection was shown to be long-lived after a single nasal vaccination [13,15]. Vaccination with BPZE1 has also recently been shown to reduce nasopharyngeal infection by *B. pertussis* in baboons by 99.992% compared to non-vaccinated baboons [16]. BPZE1 has now successfully completed a first-in-man phase I clinical trial and was found to be safe in adults, able to transiently colonize the human nasopharynx and to induce immune responses to all tested antigens in all colonized individuals [17].

The parental strain of BPZE1 is the Tohama I derivative BPSM [9]. Tohama I produces serotype 2, but not serotype 3 fimbriae [18], and many currently circulating strains produce serotype 3 fimbriae (Fim3) [19,20]. Since fimbriae may contribute to protection against *B. pertussis* [21], BPZE1 may be more effective against Fim2-producing than against Fim3-producing clinical isolates. In this study, we therefore constructed a BPZE1 derivative that produces both Fim2 and Fim3 and tested its protective potency against clinical isolates that differ with respect to their Fim2 and Fim3 production.

2. Materials and methods

2.1. Culture conditions

All *B. pertussis* strains were grown on Bordet Gengou (BG) agar with 10% (v/v) sheep blood, in modified Stainer Scholte (SS) medium under agitation as described [22] or in fully synthetic Thijs medium [23]. The media were supplemented with the appropriate antibiotics (100 mg/ml of streptomycin or 10 mg/ml of gentamycin for the strains carrying pFUS2-BctA1 [24]).

2.2. Bacterial strains

B. pertussis BPSM and BPZE1, as well as *Bordetella parapertussis* used in this study have been described previously [9,25]. *B. pertussis* strains B0403, B1412, B1617 and B0005 (strain 134 Pillmer) came from the RIVM collection (Bilthoven, The Netherlands). For counterselection purposes some of the clinical isolates were electroporated with the pFUS2-BctA1 suicide plasmid to acquire gentamycin resistance as described in [24]. After electroporation gentamycin-resistant derivatives were checked by PCR to verify the site of insertion of the pFUS2-BctA1 vector into the chromosomal DNA and by ELISA to check the level of surface-exposed Fim2 and/or Fim3, as described below. Strain P134S was obtained by selecting a streptomycin resistant derivative of *B. pertussis* B0005. In addition to streptomycin resistance mediated by a mutation in the *rpsl* gene, the strain has a mutation in the *fimC* gene leading to the loss of the fimbriae production. *E. coli* SM10 [26] was used for conjugation the various plasmid constructs into *B. pertussis*.

2.3. Construction of the Fim3-positive BPZE1-derivative BPZE1f3

To construct BPZE1f3, the 13-C stretch located in the promoter region of the *fim3* gene of BPZE1, 75 bp upstream of the *fim3* ATG codon, was replaced by a 14-C stretch in order to drive the transcription of *fim3*. The whole *fim3* locus, containing the promoter region, was first deleted from the parental strain and then replaced by a *fim3* locus with a 14-C stretch. A 2255-bp PCR fragment encompassing the locus was amplified by using the following oligonucleotides (SPfim3UP2: GAGCTCTTTACCGCGGCCGC CAGTTGTTCATCAATG and ASPfim3LO2: GGATCCATCATCGAGACC-GACTGG) and cloned into the *Sac*I and *Bam*HI restriction sites of a pBluescript II SK+ plasmid (Addgene). From the resulting plasmid, a 904-bp fragment containing the whole locus was removed by *Sph*I restriction to obtain pSKfim3UPLO. The 1355-bp *Sac*I-*Bam*HI fragment of pSKfim3UPLO was inserted into the *Sac*I and *Bam*HI sites of pJQ200mp18rpsl [24]. The recombinant plasmid was then used for double homologous recombination in BPZE1 using conjugation as described previously [9]. The transconjugants were checked for deletion of the whole *fim3* locus by PCR using oligonucleotides SPfim3UP2 and ASPfim3LO2. Reintroduction of the whole *fim3* locus with the 14-C stretch in the promoter was done as follows. A 911-bp synthetic gene encompassing the whole locus with the 14-C stretch was synthesized by GeneArt® Gene Synthesis (ThermoFisher SCIENTIFIC). *Sph*I sites at the extremities of the synthetic fragment were used to insert it into the *Sph*I site of pSKfim3UPLO giving rise to pSKfim3+. The correct orientation of the insert was checked by restriction enzyme analyses and DNA sequencing. The 2256-bp *Sac*I-*Bam*HI fragment of this plasmid was transferred into the same restriction sites of pJQ200mp18rpsl leading to pJQfim3+. This plasmid was used to perform the double homologous recombination to obtain BPZE1f3. The recombinant strain was verified by PCR using oligonucleotides SPfim3UP2 and ASPfim3LO2.

2.4. Analysis of Fim2 and Fim3 production

The *B. pertussis* strains were first inactivated at 56 °C for 30 min. The heat-inactivated strains were then coated with 100 μl per well at an optical density $(0D)_{600nm}$ of 0.075 in 96-well plates (Nunc Maxi Sorp,) and incubated overnight at 37 °C until the wells were dry. The wells were then blocked with 100 μl of PBS Tween 0.1% (PBST), containing 1% bovine serum albumin (BSA). Serial dilutions of Fim2 and Fim3 monoclonal antibodies (NIBSC, 04/154 and 04/156, respectively) were added from 1/50 to 1/36,450 in PBS Tween 0.1% (v/v). After three washes, the plates were incubated with 100 μl of horseradish-peroxidase-labelled goat anti-mouse IgG (Southern Biotech) in PBST. Following five washes, the plates were incubated with 100 μl of HRP Substrate TMB solution (Interchim) for 30 min at room temperature. The reaction was stopped by the addition of 50 μl 1 M H_3PO_4. The OD was measured with a Biokinetic reader EL/340 microplate at 450 nm.

2.5. DNA sequencing

PCR amplification of chromosomal DNA was performed using Phusion High-Fidelity DNA Polymerase (Thermofisher) or KAPA HiFi DNA Polymerase (Kapa Biosystems) according to the manufac-