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Potential use an *Actinobacillus pleuropneumoniae* double mutant strain Δ*apxIIC*Δ*apxIVA* as live vaccine that allows serological differentiation between vaccinated and infected animals

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Abstract

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia, a highly contagious and often fatal disease. We have previously reported the construction and characterization of a single gene *apxIIC* deletion mutant HB04C⁻ based on *A. pleuropneumoniae* serovar 7 which produces ApxII toxin and ApxIV. A precisely defined Δ*apxIIC*Δ*apxIVA* double-deletion mutant of *A. pleuropneumoniae* was constructed based on HB04C⁻ by transconjugation and counterselection, and the levels of virulence of the Δ*apxIIC* single mutant and Δ*apxIIC*Δ*apxIVA* double mutant were compared in an experimental infection in mice and pigs. The results demonstrated that the Δ*apxIIC*Δ*apxIVA* double mutant strain was less virulent than HB04C⁻. Despite attenuation of virulence, the Δ*apxIIC*Δ*apxIVA* double mutant remains immunogenic and conferred a similar level of protective immunity to pigs against challenge with a lethal dose of a heterologous fully virulent standard serovar 1 strain of *A. pleuropneumoniae*. The results of the virulence study suggest that ApxIV is a critical virulence factor of *A. pleuropneumoniae* serovar 7 and is able to induce clinical disease, but it not required for efficient vaccination of pigs against *A. pleuropneumoniae* infection. Two weeks after the booster immunization, animals vaccinated with HB04C⁻ were positive in the ApxIVAM-ELISA based on a recombinant GST-fusion protein GST-ApxIVAM as the solid-phase antigen while animals vaccinated with the Δ*apxIIC*Δ*apxIVA* double mutant were negative. These data demonstrate that the double mutant Δ*apxIIC*Δ*apxIVA* can be used as an effective live marker vaccine allowing serological differentiation between vaccinated and infected animals.

Keywords: *Actinobacillus pleuropneumoniae*; Double mutant; Live vaccine; Serological differential diagnosis

1. Introduction

Actinobacillus pleuropneumoniae (*A. pleuropneumoniae*; APP) is the etiological agent of porcine pleuropneumonia (PCP), a severe and often fatal respiratory disease of swine, which is associated with significant economic losses in industrialized pigs production worldwide [1]. To date, 15 serovars of *A. pleuropneumoniae* have been identified [2]. The virulence of *A. pleuropneumoniae* is associated with several factors which involved in the pathogenesis of *A. pleuropneumoniae* of PCP, such as capsular polysaccharides [3], lipopolysaccharides [4], outer membrane proteins [5], adhesion factors [6,7], proteases [8] and exotoxins [9–11]. However, the virulence of *A. pleuropneumoniae* has been found to be strongly but not exclusively correlated with the presence of Apx toxins. Four different Apx toxins have been

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found to be produced by the 15 serotypes: ApxI, ApxII, ApxIII and ApxIV [2,10,12]. The RTX toxins play a predominant role in pathogenesis, and ApxI, ApxII and ApxIII are strongly immunogenic and involved in the induction of protective immunity [11,13,14]. ApxI is strongly hemolytic and strongly cytotoxic, ApxII is weakly hemolytic and moderately cytotoxic, ApxIII is nonhemolytic but stongly cytotoxic. A fourth RTX toxin, ApxIV, reported recently, ApxIV is specific to the species of *A. pleuropneumoniae* [15], production of the ApxIVA protein has not been detected in *A. pleuropneumoniae* cultures *in vitro*. When expressed in *Escherichia coli*, recombinant ApxIVA shows weak hemolytic activity and cohaemolytic synergy with the sphingomyelinase (*beta*-toxin) of *Staphylococcus aureus*. These activities required the presence of an additional gene, ORF1, that is located immediately upstream of *apxIVA*. [12]. However, little is known about the role of ApxIV in the pathogenesis of *A. pleuropneumoniae*.

Vaccination is potentially an effective tool for the prevention of PCP. Current commercial vaccines are still primarily killed whole cell bacterins and Apx toxins-based subunit vaccines, which generally reduce mortality from APP infection but frequently fail to induce cross-serotype immunity and prevent severe morbidity [16,17]. In contrast, natural or experimental infection with a virulent strain of *A. pleuropneumoniae* generally elicits at least partially protection against reinfection with another serotype [18]. Recent advances in the genetics of *A. pleuropneumoniae* have led to the development of attenuated *A. pleuropneumoniae* vaccine strains with single or multiple defined mutations in the bacterial genome [19–23].

A major drawback of vaccination, as a disease control measure, is that the immunized animals produce antibodies against the vaccine strain and can therefore no longer be distinguished from field-exposed animals by serological tests. Deletion of a diagnostic protein from the live attenuated vaccine strain results in a mutant that does not induce antibodies towards the target protein. The protein can then be used in tests to differentiate between vaccinated animals and infected animals [24]. The *apxIVA* gene product cannot be detected in *A. pleuropneumoniae* cultures grown under various conditions *in vitro*, but the *apxIVA* gene can be detected in all 15 *A. pleuropneumoniae* serovars [25]. Therefore, deletion of *apxIVA* should allow the serological differentiation between animals immunized with the Δ*apxIVA* vaccine strain and animals infected by wild-type *A. pleuropneumoniae* strains. An ELISA test was used in this study to detect antibodies against ApxIVA-antigenic determinants of the *apxIV* gene of *A. pleuropneumoniae*. Specific antibodies against *A. pleuropneumoniae* were detected in sera of pigs inoculated with wild-type strain but not in the sera of pigs inoculated with the *A. pleuropneumoniae* Δ*apxIVA* vaccine strain. We have previously reported the construction of a single *apxIIC* gene deletion mutant based on serovar 7 [26]. The research showed that the *A. pleuropneumoniae apxIIC* mutant was attenuated but retained some residual virulence for inoculated mice and pigs. Protection against homologous and heterologous *A. pleuropneumoniae* was observed in BALB/c mice. A major drawback of the described single mutant vaccine strain, HB04C$^-$, is the fact, that vaccinated animals cannot be distinguished from naturally infected ones. Taking these factors into account, attenuated, cross-protective and phenotypically distinct mutants without inserted markers are most desirable as candidate live attenuated vaccines. In this study, we introduced another mutation into the *apxIVA*, which was selected because it has low homogeneity with other RTX toxins (except *frpA* and *frpC* of *Neisseria meningitides*), it exists in all *A. pleuropneumoniae* serotypes and it can induce high levels of antibodies after infection with wild strains. We evaluated the virulence of the double mutant strain in mice and pigs, and its immunogenic potential in mice and swine compared with its parental strain HB04C$^-$. The Δ*apxIIC*Δ*apxIVA* double-deletion mutant of *A. pleuropneumoniae* serovar 7 was more thoroughly attenuated and safer than the Δ*apxIIC* single mutant in BALB/c mice and 6-week-old pigs, and conferred the same degree of protection on immunized mice and pigs against challenge with homologous and/or heterologous serovars of *A. pleuropneumoniae*.

2. Materials and methods

2.1. Bacterial strains, plasmids, primers and growth conditions

The bacterial strains, plasmids, and primers used in this work are listed in Table 1. *Escherichia coli* strains were cultured in Luria–Bertani broth, supplemented with appropriate antibiotics (ampicillin, 50 μg/mL); for cultivation of *E. coli* β2155 (Δ*dap*), diaminopimelic acid (50 μg/mL, Sigma–Aldrich, Munich, Germany) was added. *A. pleuropneumoniae* strains were cultured in Tryptic Soy Broth (TSB) or Tryptic Soy Agar (TSA) (Difco GmbH, Augsburg, Germany) supplemented with NAD (10 μg/mL, Sigma–Aldrich), for the selection of *A. pleuropneumoniae* transconjugants, chloramphenicol (2 μg/mL) was added; sucrose (10%, v/v, Sigma–Aldrich) was added during the sucrose counterselection procedure.

2.2. Construction of the ΔapxIICΔapxIVA double mutant

A 2.7 kb fragment of the N-terminal of *apxIVA* was amplified from the genomic DNA of *A. pleuropneumoniae* single mutant Δ*apxIIC* using the primers P1 and P2 and cloned into *Sal*I-*Not*I sites of pBluescript SK(+), resulting in plasmid pSN1. Then, the 0.6 kb fragment from *apxIVA* gene in pSN1 was digested by *Bam*HI-*Sma*I and removed, and the stick end of *Bam*HI was blunt-ended by Klenow I and ligated again, resulting in plasmid pSNT1. The transconjugation plasmid pENT1 was constructed by ligation of the *Sal*I-*Not*I fragment containing the truncated N-terminal of *apxIVA* into pEMOC2.