Published in final edited form as:
Vaccine. 2010 July 12; 28(31): 4871–4874. doi:10.1016/j.vaccine.2010.04.113.

Population-based analysis of *Actinobacillus pleuropneumoniae* ApxIVA for use as a DIVA antigen

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Abstract

APXIVA is an RTX toxin of *Actinobacillus pleuropneumoniae* that is a candidate antigen to differentiate infected from vaccinated animals (DIVA). Insertion of IS*Apl1* into the *apxIVA* gene is known to compromise an APXIVA-based DIVA approach, as is potentially a TGG to TGA mutation in the *apxIVA* gene. IS*Apl1* was found in 63/349 (18.1%) *A. pleuropneumoniae* isolates from England and Wales including serovars 2, 3, 6–8 and 12. No IS*Apl1* insertions into *apxIVA* were found. Only two serovar 3 isolates contained the TGG to TGA mutation. We conclude that an ApxIVA-based DIVA approach would potentially be viable in England and Wales.

Keywords

Actinobacillus pleuropneumoniae; DIVA; ApxIVA; Vaccine; Serotyping

1 Introduction

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia, a highly contagious economically important endemic bacterial disease of pigs worldwide [1]. Following infection via aerosols or pig to pig contact, acute disease may manifest as severe respiratory distress that can lead to death 24–48 h later. Alternatively, chronic infection may occur, where the bacterium causes a sub-clinical pleuropneumonia that may only be detected in the abattoir. Vaccination is considered the best approach to prevent infection [2,3].

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Protection by bacterins, the first generation vaccines for *A. pleuropneumoniae* infection, which are still widely used, is usually associated with a reduced mortality rate but lacks some important features. It is generally only serovar-specific (homologous), and bacterins inconsistently prevent colonisation or the development of lung lesions [3,4]. Second generation vaccines have been based on the RTX toxins of *A. pleuropneumoniae*, e.g. Porcillis APP (Intervet). However, none of the commercialised subunit vaccines provide complete protection against *A. pleuropneumoniae* infection[3]. Additionally, the discovery of further subunit vaccines is hampered because surface-exposed virulence factors or proteins that are targets of the immune response may only be expressed in vivo [3]. Partly because of the problems indicated above, currently, there is a move towards the development of live attenuated vaccines, which have advantages including: low effective doses (since they are self-replicating) and they closely mimic natural infection resulting in immune responses at the relevant body site. In the case of *A. pleuropneumoniae*, live attenuated vaccines can protect not only against the homologous but also heterologous serovars [5–7]. One *A. pleuropneumoniae* live attenuated vaccine, which contains a serovar 1 capsule mutant, is now available in North America (Ingelvac, APP-ALC, Boehringer Ingelheim) but not the rest of Europe, Asia or Africa. From a regulatory and practical standpoint, there is a great advantage in being able to identify animals which have been vaccinated from those that are colonised or have chronic disease. This is the "Differentiating Infected from Vaccinated Individuals (DIVA) principle [8,9], which can be achieved for live attenuated vaccines by inactivation of a gene encoding an antigenic protein so that antibodies are no longer elicited against that marker protein. Simple tests such as serum-based ELISAs can then be used to differentiate between vaccinated and non-vaccinated infected animals.

The *apxIVA* gene is present in all *A. pleuropneumoniae* strains and is species-specific, hence its use to confirm identification of the organism [10,11]. ApxIVA should be an excellent antigen for use in a DIVA approach, since it is produced in vivo by all serovars and is immunogenic both in animals that have had clinical and those that have had sub-clinical disease [12]. Inactivation of *apxIVA* in the vaccine strain should permit serological differentiation between infected and vaccinated pigs. This has been supported by Liu et al. [13] using a live attenuated vaccine strain consisting of a serovar 7 double mutant in which *apxIIC* and *apxIVA* had been deleted. The double mutant was less virulent in pigs than a single *apxIIC* mutant, and conferred high level protection against a lethal dose of a heterologous serovar 1 strain. Two weeks after a booster immunisation, pigs that had received the *apxIICapxIVA* double mutant were serologically negative in an ApxIVA ELISA in contrast to those receiving the *apxIIC* mutant, which were seropositive. Thus the *apxIICapxIVA* double mutant was successful as a live attenuated vaccine and *apxIVA* deletion allowed the differentiation between vaccinated and infected animals.

However, it is important that this promise translates from the laboratory to the field. It would be unwise to invest time and money in ApxIVA as a DIVA antigen if there are significant obstacles to its widespread use: specifically, if any *A. pleuropneumoniae* field isolates do not produce ApxIVA in vivo, or if non-*A. pleuropneumoniae* isolates produce the toxin. Recent work suggesting that there may be cause for concern over the use of ApxIVA as a DIVA antigen comes with the description of a new mobile genetic element (IS*Apl1*) capable of insertion into the *apxIVA* gene. IS*Apl1* was found to have inserted into the *apxIVA* gene in 5

out of the 23 serovar 7 strains investigated including AP76 [14]. Convalescent sera from pigs experimentally infected with AP76 were sero-negative for ApxIVA, demonstrating that IS*Apl1* insertion had resulted in loss of ApxIVA expression in vivo. Only small numbers of other serovars were investigated for evidence of IS*Apl1* [14].

Additionally, a Trp to stop (TGG to TGA) mutation has been described in the coding region of the *apxIVA* gene (APJL1016) in the genome of the serovar 3 isolate JL03 (accession number: CP000687) [15]. The mutation is predicted to result in a truncated protein of 133 rather than 1570 amino acids. The same mutation was present in one out of three serovar 3 clinical isolates and in the reference strain S1421 [15]. In the case of S1421, the mutation does not appear to affect expression of ApxIVA in vivo since experimental infection of pigs with this strain resulted in the production of antibodies recognising both N- and C-terminal parts of the protein [16]. However, it remains a possibility that the mutation could be associated with loss of APXIVA expression in other isolates. The aims of this study were to determine (1) the extent of IS*Apl1* insertion into *apxIVA* and (2) the frequency of the TGG to TGA mutation within *apxIVA* in clinical isolates from England and Wales. The results would determine whether an approach using ApxIVA as a DIVA antigen would be feasible in these countries.

2 Materials and methods

2.1 Bacteria, growth and serotyping

A. pleuropneumoniae isolates ($N = 349$) from England and Wales were obtained from the Veterinary Laboratories Agency (VLA, Bury St Edmunds) having been collected between 1995 and 2009. A single serovar 3 isolate from England and nine from Switzerland [17,18] were obtained from our non-VLA collection. All isolates were *apxIVA*-positive [18,21]. Bacteria were grown on Brain Heart Infusion agar plus Levinthal's base at 37 °C in 5% carbon dioxide [23].

PCRs for the identification of serovar 2 [19] and serovars 7 and 12 [20] were as described except that HotStarTaq and supplied buffer (Qiagen) were used throughout. Serovar 3, 6 and 8-*apxIVA* multiplex PCR was carried out as described previously [18,21]. The DNA template was either cellular lysate or that isolated using the QIAamp kit (Qiagen). Appropriate *A. pleuropneumoniae* reference strains were used as positive and negative controls throughout.

2.2 PCR screening for the presence of ISApl1 and insertion into apxIVA and other genes

PCR screening was used to determine for the presence of the insertion element, IS*Apl1* within a genome, and, for those that contained IS*Apl1,* subsequently for insertion of the element into the *apxIVA*, *ataC*, *bla* or *cps* genes, as previously described [14]. *A. pleuropneumoniae* serovar 7 isolate AP76 was used as the positive control [14].

2.3 DNA-sequencing to determine the presence or absence of the TGG to TGA mutation in apxIVA

The frequency of the TGG to TGA mutation in *apxIVA* [15] within the *A. pleuropneumoniae* population was determined in representative isolates. This was achieved by Big Dye DNA-sequencing (Applied Biosystems) of a 1153 bp PCR amplicon containing the potentially mutated site using the primers pSapxIVA 5′-AGTTGGAGTCAATGCTCCAAACAAC-3′ and pRapxIV 5′-CAGCGAGAGGGCAAAGCGTA-3′ designed to amplify a fragment of the *apxIVA* gene within the *A. pleuropneumoniae* serovar 3 JL03 genome. The PCR conditions were as follows: 95 °C for 5 min followed by 95 °C for 1 min, 54 °C for 1 min, and 72 °C for 1 min 15 s (32 cycles) and 72 °C for 10 min. Genomic DNA (10 ng) was used as template. Primers were used at a concentration of 0.5 μM, and used for both PCR amplification and DNA-sequencing. However, a PCR amplicon was not obtained from every isolate. Therefore, primers were redesigned, based on conserved nucleotide sequences present in the *apxIVA* genes in the *A. pleuropneumoniae* serovar 3, 5b (accession number: CP000569) and 7 (accession number: CP001094) genomes, to amplify a 500 bp PCR amplicon. The PCR conditions were as detailed above except that the primers ApxIVseq_F 5′-CCG GGC AAA TAT TCC AAA GCG CAG-3′ and ApxIVseq_R 5′-TTC CAC CTC GCC GGT AAA GTT AAG G-3′ were used. Representative IS*Apl1* positive and negative isolates were screened.

3 Results

3.1 Presence of ISApl1 in *A. pleuropneumoniae* isolates from England and Wales

IS*Apl1* was present in 63/349 (18.1%) isolates. There were IS*Apl1* insertions in serovar 2 (3/11, 27.3%), serovar 3 (1/1, 100%), serovar 6 (7/24, 29.2%), serovar 7 (4/20, 20.0%), serovar 8 (44/283, 15.5%) and serovar 12 (4/10, 40.0%) isolates.

3.2 No isolates had ISApl1 inserted into their apxIVA gene

All of the isolates possessing IS*Apl1* ($N = 63$) were screened by PCR to determine whether the insertion element had interrupted *apxIVA* as had been reported previously [14]. All 63 isolates produced a PCR amplicon of the predicted wild-type size; not one had an insertion into the *apxIVA* gene. In some serovar 7 isolates, IS*Apl1* was found in locations in the chromosome other than *apxIVA* [14]. Twenty two randomly chosen isolates (serovar 2, 6, 7, 8 and 12) were screened by PCR for the presence of IS*Apl1* into the *ataC*, or *bla* and *cps* genes as described previously [14]. One insertion was found in the *bla* and one in the *cps* gene in single isolates of serovar 6 and 7, respectively.

3.3 The TGG to TGA mutation in apxIVA is only present in *A. pleuropneumoniae* serovar 3 isolates

DNA-sequencing showed that the mutation was absent from the 22 random serovar 2, 6, 7, 8 and 12 (both IS*Apl1* positive and negative) isolates tested. However, the mutation was found to be present in 10/10 serovar 3 isolates present in our non-VLA collection. These comprised one additional isolate from England and a set of nine Swiss isolates which had

previously been used to formulate toxin-based typing schemes [17], and in the validation of serovar 3 [22] and serovar 3, 6 and 8 [18] PCRs.

4 Discussion

IS*Apl1* was present in 18.1% isolates from England and Wales but was not confined to serotype 7 as had been previously reported [14]. Instead it was present in serovar 2, 3, 6–8 and 12 isolates indicating that it is more widespread than previously recognised. No isolate had insertion of IS*Apl1* into its *apxIVA* gene, although insertion was found within the *bla* and *cps* genes of single isolates of serovar 6 and 7, respectively. Thus, IS*Apl1* inserts additionally into different sites other than those that are known. Our data suggest that the site of IS*Apl1* insertion into *apxIVA* as reported previously [14] is not an insertion hot spot. *A. pleuropneumoniae* is a naturally transformable bacterium [23], but there is also no evidence in our study suggesting widespread transfer of IS*Apl1* in *apxIVA* through the natural population via this route.

The TGG to TGA mutation in *apxIVA* was only found in serovar 3 isolates. It has now been described in isolates from diverse origins (China, Switzerland and England). Further, more comprehensive, studies will be required to determine whether the mutation is serovar 3 specific. Serovar 3 isolates have been considered as low virulence [24]. Inactivation of *apxIVA* in a serovar 7 isolate resulted in attenuation, suggesting ApxIVA is important for virulence in vivo [25]. It is possible that, at least in part, the low virulence of serovar 3 isolates may be due inactivation of the *apxIVA* by the TGG to TGA mutation, although the production of ApxIVA antibodies after experimental infection of the serovar 3 reference strain S1421, which harbours the mutation (33), argues against this. Transcription of the C-terminal portion of ApxIVA in S1421 most likely occurs via an in-frame ATG immediately downstream of the TGG to TGA mutation.

Our data, based on analysis of 350 *A. pleuropneumoniae* isolates from England and Wales ($N = 349$ from the VLA collection plus one additional serovar 3 English isolate from the non-VLA collection), indicate that an ApxIVA-based DIVA approach would not be compromised in a single case. This is based on the assumption that the TGG to TGA mutation in *apxIVA* does not affect ApxIVA expression in vivo, as the experimental evidence suggests. Even if the mutation did affect ApxIVA expression then an ApxIVA-based DIVA approach would be predicted to be compromised in only 2/350 cases (0.6%), each of which involve low-virulence serovar 3 isolates. Thus, the data in this study indicate that *apxIVA* inactivation in field isolates is a rare event. It should be emphasised that only known or theoretical inactivation mechanisms have been investigated. It is possible that other mechanisms of inactivation of *apxIVA* exist. These might include mutations in the *apxIVA* promoter region, the presence of IS*Apl1* (or other insertion elements) in other regions of the *apxIVA* gene or mutations in transcriptional regulators of *apxIVA* expression in vivo. None of these has been described to date.

We conclude that an ApxIVA-based DIVA approach is potentially viable but recommend a confirmation of its likeliness to being effective before being introduced in a similar manner to that carried out with UK isolates in this study. It is most likely that an ApxIVA-based

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DIVA approach would be used in combination with other additional attenuating mutation(s). Re-evaluation will be required if any other mechanism(s) are found that compromise an ApxIVA-based DIVA approach.

Acknowledgments

This study was supported by the Biology and Biotechnology Science Research Council (PRL, ANR, JSK). CON was supported by The Wellcome Trust. We are grateful to Dr Zuofei Xu and Professor Rui Zhou for supplying details of primers and PCR conditions enabling identification of isolates with a mutation in their *apxIVA* gene, and Professor Joachim Frey (University of Berne) for supplying serovar 3 isolates.

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