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Predicting Genetic Traits and Epitope Analysis of *apxIVA* in *Actinobacillus pleuropneumoniae*

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Actinobacillus pleuropneumoniae **causes a severe hemorrhagic pneumonia in pigs. Fifteen serotypes of *A. pleuropneumoniae* express four different Apx toxins that belong to the pore-forming repeats-in-toxin (RTX) group of toxins. ApxIV, which is conserved and up-regulated *in vivo*, could be an excellent candidate for the development of a protective cross-serotype immunity vaccine, and could aid in the differential diagnosis of diseases caused by *A. pleuropneumoniae*. We identified and sequenced *apxIVA* from *A. pleuropneumoniae* serotype 2 isolated in Korea (Kor-ApxIVA). The Kor-ApxIVA was closely related to Switzerland (AF021919), China (CP000687), and China (GQ332268), showing 98.6%, 98.4%, and 97.2% amino acid homology, respectively. The level of amino acid homology, however, was higher than the nucleotide homology. The structural characteristics of ApxIVA showed RTX proteins, including N-terminal hydrophobic domains, signature sequences for potential acylation sites, and repeated glycine-rich nonapeptides in the C-terminal region of the protein. Thirty glycine-rich nonapeptides with the consensus sequence, L/V-X-G-G-X-G-N/D-D-X, were found in the C-terminus of the Kor-ApxIVA. In addition, the Kor-ApxIVA was predicted for the linear B-cell epitopes and conserved domains with determined peptide sequences. This genetic analysis of the Kor-ApxIVA might be an important foundation for future biological and functional research on ApxIVA.**

Keywords: *Actinobacillus pleuropneumoniae*, RTX toxins, ApxIV, sequences

Actinobacillus pleuropneumoniae causes a highly contagious pleuropneumonia in pigs, leading to major economic losses in pig farming worldwide (Nielsen, 1988; Fenwick and Henry, 1994; Frey, 1995; Haesebrouck *et al.*, 1997). *A. pleuropneumoniae* can result in various clinical degrees of pleuropneumonia from peracute to chronic, with infected pigs typically showing a hemorrhagic, necrotizing pneumonia often associated with fibrinous pleuritis (Haesebrouck *et al.*, 1997). The virulence of *A. pleuropneumoniae* is multifactorial, and the factors involved in pathogenesis are capsular polysaccharides, lipopolysaccharides, outer membrane proteins, adhesion factors, proteases, and exotoxins (Udeze *et al.*, 1987; Dom *et al.*, 1994; Tascon *et al.*, 1994; Frey, 1995; Haesebrouck *et al.*, 1997; Baltes *et al.*, 2002; Bandara *et al.*, 2003; Negrete-Abascal *et al.*, 2003). However, the respective contributions of the virulence factors to the infection and disease are not known. Among these factors, exotoxins have been reported to be strongly related to pathogenesis, and indicate the virulence of the different serotypes (Tascon *et al.*, 1994; Frey, 1995; Haesebrouck *et al.*, 1997; Kamp *et al.*, 1997; Bosse *et al.*, 2002; Boekema *et al.*, 2004). Fifteen serotypes express four different Apx toxins belonging to the pore-forming repeats-in-toxin (RTX) toxins (Haesebrouck *et al.*, 1997; Blackall *et al.*, 2002). ApxI is strongly hemolytic and cytotoxic, while ApxII is weakly hemolytic and moderately cytotoxic. However, ApxIII is nonhemolytic, but strongly cytotoxic (Frey, 1995; Schaller *et al.*, 1999). Recombinant ApxIV shows weak hemolytic activity and cohemolytic synergy with sphingomyelinase (*beta-toxin*) of *Staphylococcus aureus*, but the biological and functional characteristics of ApxIV are unclear (Schaller *et al.*, 1999). ApxI, ApxII, and ApxIII are also produced by other *Actinobacillus* species such as *A. rossii*, *A. suis*, and *A. porcitonsillarum* (Schaller *et al.*, 2001; Gottschalk *et al.*, 2003), whereas ApxIV is highly specific to *A. pleuropneumoniae* (Schaller *et al*., 1999).

The Apx toxin is encoded by the *apx* operon, which contains four genes in the order *apxCABD*: the activator gene, *apxC*, the pretoxin structural gene, *apxA*, and the secretion apparatus-encoding genes, *apxB* and *apxD* (Frey, 1995). *ApxIV* has a similar sequence to the *frpA* and *frpC* genes of *N. meningitidis*, and is located immediately downstream of the *A. pleuropneumoniae lacZ* gene and upstream of the ORF1 gene (Anderson and MacInnes, 1997; Schaller *et al.*, 1999). ORF1 seems to be involved in activation and is required for the observed hemolytic and cohemolytic (CAMP) phenotypes (Schaller *et al.*, 1999).

Compared to ApxI, ApxII, and ApxIII, ApxIV has distinctive features that are produced by all serotypes of *A. pleuropneumoniae*. These features are highly specific to *A. pleuropneumoniae* and are expressed only *in vivo* (Schaller *et al.*, 1999; Cho and Chae, 2001; Schaller *et al.*, 2001; Dreyfus *et al.*, 2004; Turni and Blackall, 2007). An ApxIV-based serological test was developed as a differential diagnostic tool to identify infected or carrier pigs (Dreyfus *et al.*, 2004). Furthermore, a subunit vaccine composed of recombinant ApxI, ApxII, ApxIII, the N-terminal half of ApxIV, as well as the recombinant outer membrane protein (OMP) of *A. pleuropneumoniae* showed strong protection against *A. pleuropneumoniae* infection.

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Table 1. Nucleotide sequence of primers used in sequencing

Primer name	Primer sequences (5′→3′)	Position	PCR parameters[a]
mrp-F	GTGGCGAAGAAATACGGTACTAAAGT	*mrp* 241-*apxIVA* 120	94, 57, 72 20 sec, 10 sec, 60 sec
orf-R	AATATCATCATCTCCTTTTCCTATTTCAG		
apxIVAN-F	CACCATGACAAAATTAACTATGCAAGA	*apxIVA* 1-1500	94, 55, 72 20 sec, 10 sec, 90 sec
apxIVAN-R	CTAACTTTTTAACTTTTTAACGGCGG		
apxIVAN#A	AGAAATAAAAGAGGTTGAAAAGGGG	*apxIVA* 1320-3517	94, 53, 72 20 sec, 10 sec, 60 sec
apxIVAN#A-R	GTCGAAACTTCACTTCCGCAT		
ApxIVADWN-L[b]	CACCGCGAAACAATTCGAAGGG	*apxIVA* 2980-5766	94, 65, 72 20 sec, 10 sec, 240 sec
ApxIVA-1R[b]	CTAGGCCATCGACTCAACCAT		
lacZ-F	CGTTTATCGAATGAGCAAACGT	*apxIVA* 5704-*lacZ* 70	94, 53, 72 20 sec, 10 sec, 30 sec
lacZ-R	ATATCGCTTAATGGAAAGCGAA		

[a] Temperatures are tabulated in the first row in degrees Celsius, and the corresponding times are in the second row
[b] Adapted from Schaller *et al*. (1999)

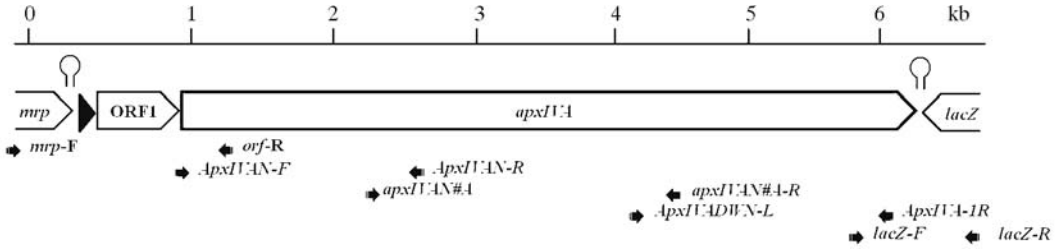

Fig. 1. Schematic of *apxIVA* and the location of *apxIVA* fragments for sequencing. Boxes with pointed tips indicate *mrp*, ORF1, *apxIVA*, and *lacZ* genes; hairpins indicate putative *rho*-independent transcription termination signals; the black triangle indicates the location of putative promoter sequences; the positions of the primer sequences used for sequencing are indicated by black arrows below the maps.

Therefore, it may be inferred that ApxIV plays a positive role in immunoprotection (Wang *et al.*, 2009). However, ApxIV itself cannot induce sufficient immune protection against *A. pleuropneumoniae* infection (Wang *et al.*, 2009).

In the present study, we described the complete sequencing and organization of the ApxIVA isolated in Korea (Kor-ApxIVA), and the homology of *apxIVA* to other *A. pleuropneumoniae* serotypes. The Kor-ApxIVA was analyzed and characterized using determined amino acid sequences. We suggest that the genetic analysis of Kor-ApxIVA is an important foundation for future biological and functional research on ApxIVA.

Materials and Methods

Bacterial strains and growth conditions

A. pleuropneumoniae KSID serotype 2 isolated from infected Korean pigs with porcine pleuropneumonia was used to investigate the *apxIVA* gene. *A. pleuropneumoniae* was grown in Luria-Bertani (LB) broth (Difco Laboratories, USA) with 0.01% β-nicotinamide adenine dinucleotide (NAD) for 24 h at 37°C.

PCR amplification

Total genomic DNA of *A. pleuropneumoniae* KSID serotype 2 was isolated using a GenElute™ Bacterial Genomic DNA kit (Sigma, USA) and subsequently used for PCR gene amplification. PCR was conducted with the following components: 1 μl of genomic DNA template, 5 μl of 10× PCR buffer (20 mM Mg^{2+}, Intron Biotechnology Inc., Korea), 5 μl of 10 mM dNTP mixture (2.5 mM of each: dATP, dCTP, dGTP, and dTTP, Intron Biotechnology Inc.), 1 μl of external forward primer (10 pmol/μl), 1 μl of external reverse primer (10 pmol/μl), 1 μl of i-*Taq* polymerase (5 units/μl, Intron Biotechnology Inc.), and 36 μl of triple-distilled H_2O in a total volume of 50 μl. Primer sequences for *apxIVA* (Table 1) were designed based on *A. pleuropneumoniae apxIVA* from Switzerland (GenBank accession no. AF021919). Amplification products were analyzed using gel electrophoresis with ethidium bromide-stained gels.

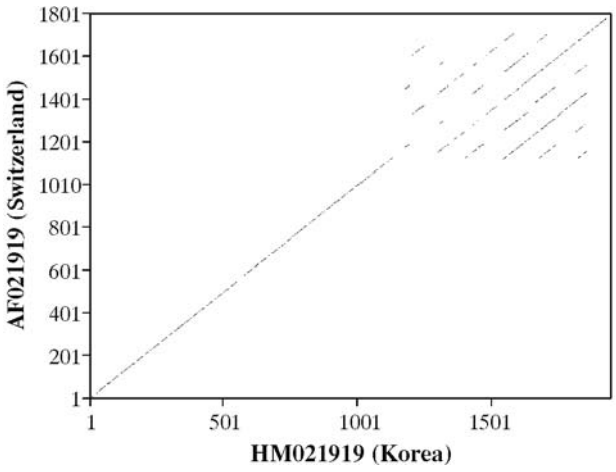

Fig. 2. A dotplot analysis of the Kor-ApxIVA (HM021919) with the GenBank accession no., AF021919, from Switzerland. A long diagonal line represents the conserved region in the N-terminus of ApxIVA. The C-terminal region of ApxIVA showed repeated regions (parallel diagonal lines); this was derived from EMBOSS server.

Table 2. Nucleotide and amino acid similarity of the Kor-ApxIVA (HM021153) with the six different GenBank accession nos. of ApxIVA according to Martinez/Needleman-Wunsch DNA alignment and Lipman-Pearson protein alignment (MegAlign 5.0 DNASTAR)

Selected strains	Similarity index of ApxIVA					
	Switzerland (AF021919)	Switzerland (AF030511)	Germany (CP001091)	Canada (CP00569)	China (CP000687)	China (GQ332268)
Nucleotide Korea (HM021153)	78.9	75.8	82.6	84.9	76.1	74.4
Amino acid Korea (HM021153)	98.6	96.7	94.7	95.8	98.4	97.2

DNA sequencing and homology analysis

Amplified *apxIVA* genes were purified with a QIAquick Gel Extraction kit (QIAGEN, Germany). The purified fragments were sequenced using automatic dye terminator DNA sequencing (ABI PRISM 377 L, Perkin Elmer, USA). The sequence data were assembled and analyzed using MegAlign 5.0 DNASTAR. Homology and the conserved domain for *apxIVA* were examined using the BLAST at the National Center for Biotechnology Information. The GenBank nucleotide sequence accession numbers of the *apxIVA* that were analyzed are as follows: AF021919, AF030511, CP001091, CP000569, CP000687, and GQ332268; the sequence accession numbers of *apxIVA* differed substantially in length. The Martinez/Needleman-Wunsch method and Lipman-Pearson method were employed to generate DNA and polypeptide alignments using MegAlign 5.0 DNASTAR. Phylogenetic trees of *apxIVA* were constructed via bootstrap analysis (500 repeats) using MEGA 4.0.

Predicting protective linear B-cell epitopes and the conserved domain

Epitope prediction and analysis were performed using Chou-Fasman Beta-Turn Prediction, Emini Surface Accessibility Prediction, Karplus and Schulz Flexibility Prediction, Kolaskar and Tongaonkar Antigenicity, Parker Hydrophilicity Prediction, and Linear Epitope Prediction. The dataset comprised of protective linear B-cell epitopes was derived from the Immune Epitope Database (IEDB). The determined amino acid sequences were found to contain conserved domains and functional annotations of the Kor-ApxIVA. It was derived from the NCBI's Conserved Domain Database (CDD).

Nucleotide sequence accession number

The nucleotide sequences of the *A. pleuropneumoniae* KSID serotype 2 *apxIVA* gene were given in GenBank under the accession number, HM021153.

Results

Genetic organization of apxIVA

A consensus 5,856 bp DNA product was obtained and confirmed by nucleotide sequencing. The Kor-ApxIVA was identified through amino acid sequences with a predicted molecular mass of 218,450.05 Da and an isoelectric point of 4.60. The Kor-ApxIVA was preceded upstream by ORF1 and the

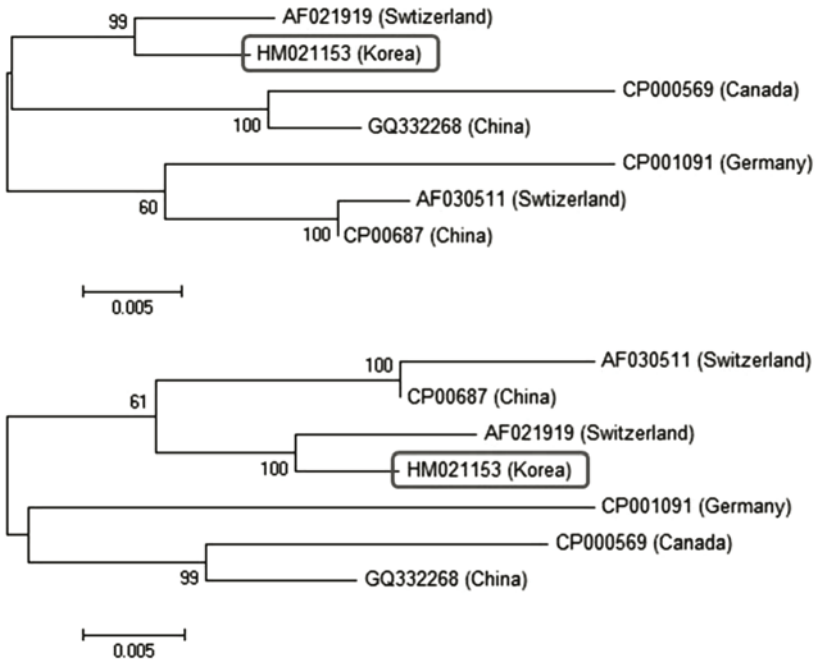

Fig. 3. Phylogenetic analysis of ApxIVA. The phylogenetic analysis was performed based on nucleotide (A) and amino acid sequences (B) compared with the Kor-ApxIVA (HM021153) and the six different GenBank accession nos. using the MEGA 4.0 program.

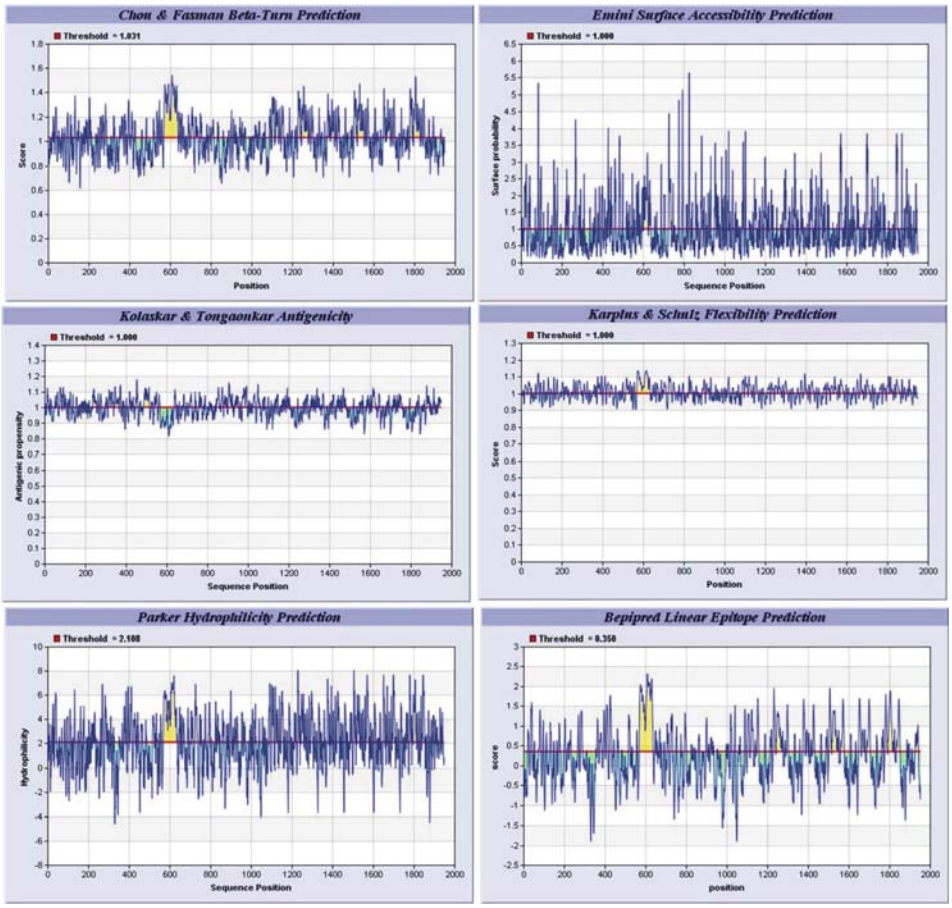

Fig. 4. Protein sequence analysis of the Kor-ApxIVA (HM021153) including beta-turn, surface accessibility, antigenicity, flexibility, hydrophilicity and linear epitope prediction. Analysis was performed using Chou-Fasman Beta-Turn Prediction, Emini Surface Accessibility Prediction, Karplus and Schulz Flexibility Prediction, Kolaskar and Tongaonkar Antigenicity, Parker Hydrophilicity Prediction, and Linear Epitope Prediction; this analysis was derived from Immune Epitope Database (IEDB).

mrp gene. *LacZ* was present in the C-terminal part of *apxIVA* (Fig. 1). A 474 bp ORF1 lay within the region between the C-terminal end of the *E. coli* methionine-rich protein, MRP (*mrp* gene product), and *apxIVA*. ORF1 encoded a protein of 157 amino acids with a molecular mass of 18,733.32 Da and an isoelectric point of 6.38. The regions of similarity between the Kor-ApxIVA (HM021153) and the GenBank accession no. (AF021919) were represented by a dotplot that displays a wordmatch of two sequences (Fig. 2). The N-terminus of ApxIVA was highly conserved, while the repeats were found at the C-terminus of ApxIVA.

Homology of ApxIVA

The Kor-ApxIVA showed homology with the *apxIVA* GenBank accession nos.: Switzerland (AF021919), Switzerland (AF030511), Germany (CP001091), Canada (CP00569), China (CP000687), and China (GQ332268) (Table 2 and Fig. 3). The nucleotide and amino acid sequences of the Kor-ApxIVA were similar to the GenBank accession nos. In particular, the Kor-ApxIVA was closely related to Switzerland (AF021919), China (CP 000687), and China (GQ332268), showing 98.6%, 98.4%, and 97.2% amino acid homology, respectively, which was higher than the nucleotide homology.

Predicting protective linear B-cell epitopes

Beta-turn, surface accessible, antigenic, flexible, hydrophilic, and linear epitope regions were distributed in the general region of the Kor-ApxIVA (Fig. 4). In addition, at the peptide position, which ranged from 568 to 636, high values were measured in the beta-turn, flexibility prediction analysis, surface accessibility, and hydrophilicity.

Structural features of the Kor-ApxIVA

The three different repeated modules constructed the C-terminal region of ApxIVA in variable sizes (Fig. 5). The C-terminus of the Kor-ApxIVA contained 30 glycine-rich nonapeptides with the consensus sequence, L/V-X-G-G-X-G-N/D-D-X. Five regions of the consensus sequences for DNA polymerase family 2 signatures, which are considered to be involved in Mg^{2+} binding (Argos, 1988), were distributed in the interval of the glycine-rich nonapeptides. The conserved domain RTX C-terminal domain (pfam08339), peptidase M10 serralysin C terminal domain (pfam08548), and multi-domain RTX toxins and related Ca^{2+}-binding proteins (COG

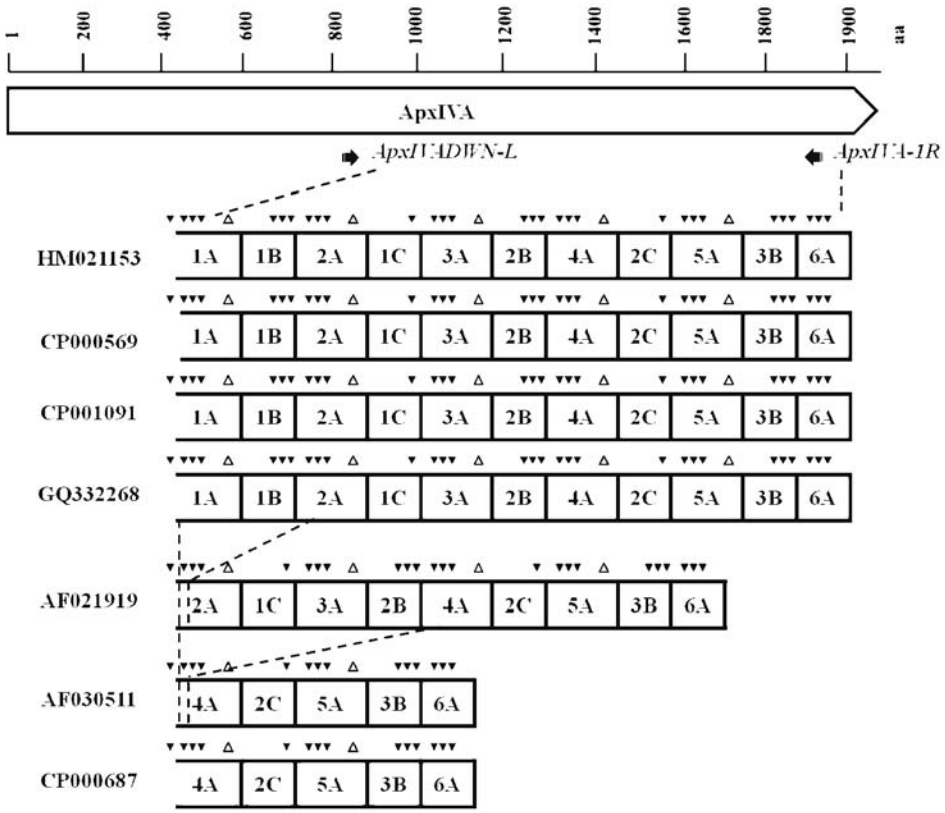

Fig. 5. Predicted structures of the Kor-ApxIVA (HM021153) and the GenBank accession nos. of ApxIVA. The black arrows with pointed tips in the lower part of the boxes indicate the oligonucleotide primers that amplified the C-terminal region of ApxIVA. The repeated glycine-rich nonapeptides are shown by filled triangles, and the open triangles indicate the positions of the DNA-polymerase family 2 signature sequences. The boxes 1A-6A, 1B-3B, and 1C-2C represent the three different repeated modules that construct the C-terminus of ApxIVA. Dotted lines indicate the site of the deletion within the repeated module, as compared to the GenBank accession nos. of ApxIVA.

2931) were shown in the C-terminal region of ApxIVA (Fig. 6). The Kor-ApxIVA (HM021153) and the GenBank accession nos. of ApxIVA had a peptidase M10 serralysin C-terminal domain extending from aa 1,089 to aa 1,135, and the RTX C-terminal domain at the C-terminus of ApxIVA. A peptidase M10 serralysin C-terminal domain was repeated in the Kor-ApxIVA (HM021153) extending from aa 1,500 to aa 1,552.

Discussion

Several current studies have focused on identifying genes that are expressed *in vivo* during a natural infection, because these genes may be important in the infection process (Fuller *et al.*, 1999; Deslandes *et al.*, 2010). These genes are thought to be regulated in the host environments encountered by the pathogen, such as conditions of iron-deficiency or a limited number of anaerobic or branched chain amino acids (BCAA) (Baltes and Gerlach, 2004; Wagner and Mulks, 2006; Deslandes *et al.*, 2007, 2010; Wagner and Mulks, 2007; Lone *et al.*, 2009). For *A. pleuropneumoniae*, the up-regulation of many genes has been reported *in vivo* (Fuller *et al.*, 1999; Deslandes *et al.*, 2010). At first, ApxIV was detected only *in vivo*, but recently its expression was discovered *in vitro* following its addition to broncho-alveolar lavage fluid (BALF) (Lone *et al.*, 2009). Although ApxIV was identified nearly a decade ago, its role in virulence and infection remain unclear (Schaller *et al.*,

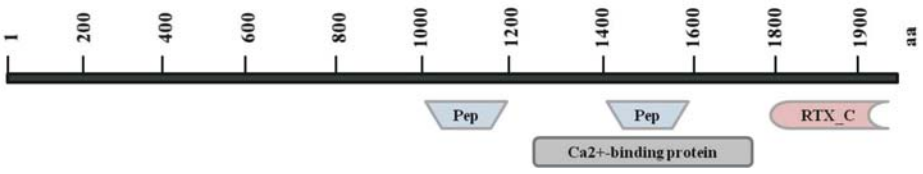

Fig. 6. Conserved domain of ApxIVA (HM021153). Pep, peptidase M10 serralysin C-terminal domain (pfam08548); RTX_C, RTX C-terminal domain (pfam08339); and Ca^{2+} binding protein, RTX toxins, and related Ca^{2+}-binding proteins.

1999).

The gene encoding ApxIVA from *A. pleuropneumoniae* serotype 2 KSID was amplified, sequenced, and characterized. The nucleotide and amino acid sequences of the Kor-ApxIVA were similar to the GenBank accession nos. of ApxIVA. The Kor-ApxIVA was homologous with the GenBank accession nos. of ApxIVA, Switzerland (AF021919), Switzerland (AF030511), Germany (CP001091), Canada (CP00569), China (CP000687), and China (GQ332268). In particular, the Kor-ApxIVA showed a higher level of homology with Switzerland (AF021919), China (CP000687), and China (GQ332268) than with the other isolates. The structural characteristics of ApxIVA show RTX proteins, including N-terminal hydrophobic domains, signature sequences for potential acylation sites, and repeated glycine-rich nonapeptides in the C-terminal region of the protein (Welch, 1991; Schaller *et al.*, 1999). The RTX family of cytotoxins are related to the pore-forming protein toxins in many Gram-negative pathogens: the a-hemolysin of *Escherichia coli* (HlyA), the bi-functional adenylate cyclase hemolysin of *Bordetella pertussis* (CyaA), the leucotoxins of *Pasteurella hemolytica* (LktA) and *Actinobacillus actinomycetemcomitans* (AaltA), as well as two iron-regulated exoproteins (FrpA, FrpC) of *Neisseria meningitidis* (Felmlee *et al.*, 1985; Lo *et al.*, 1987; Glaser *et al.*, 1988; Lally *et al.*, 1989; Schaller *et al.*, 1999). The RTX family has tandem-repeat, glycine-rich nonapeptides with the consensus sequence, Leu/Ile/Phe-Xaa-Gly-Gly-Xaa-Gly-Asn/Asp-Asp-Xaa (Schaller *et al.*, 1999). The glycine-rich nonapeptide repeats, and DNA polymerase 2 signature sequences, which are known to bind Ca^{2+} and Mg^{2+}, respectively, are organized together with nonapeptide repeats as a modular structure in the C-terminal region (Argos, 1988; Schaller *et al.*, 1999). Thirty glycine-rich nonapeptides with the consensus sequence, L/V-X-G-G-X-G-N/ D-D-X, were found in the C-terminus of the Kor-ApxIVA. An important characteristic of ApxIVA is its various sizes resulting from the different numbers of repeated modules in each serotype, and this characteristic could be used as a reliable method for typing *A. pleuropneumoniae* (Sthitmatee *et al.*, 2003; da Costa *et al.*, 2004; Rayamajhi *et al.*, 2005).

The conserved domain RTX C-terminal domain (pfam 08339), peptidase M10 serralysin C terminal domain (pfam 08548), and multi-domain RTX toxins and related Ca^{2+}-binding proteins (COG2931) were found in the C-terminal region of the Kor-ApxIVA. The RTX C-terminal domain (pfam08339) describes the C-terminal region of various bacterial hemolysins and leukotoxins (Czuprynski and Welch, 1995). The peptidase M10 serralysin C terminal region (pfam08548) is considered to be important for secretion of the protein through the bacterial cell wall, which contains the calcium ion-binding domain, pfam00353. This domain is repeated in the C-terminus of the Kor-ApxIVA, and is thought to affect the secretion of the Kor-ApxIVA. However, further experiments are needed to confirm this effect compared to other strains. In addition, the multi-domain RTX toxins and related Ca^{2+}-binding proteins conserved domain (COG2931) encode secondary metabolite biosynthesis, transport, and catabolism (Coote, 1992). Mapping B-cell epitopes is important for vaccine design, immunodiagnostic tests, and antibody production (El-Manzalawy *et al.*, 2008). The resulting dataset comprised of B-cell epitopes is believed to closely approximate a dataset of protective linear B-cell epitopes (Sollner *et al.*, 2008). Since the experimental determination of the B-cell epitopes is exhaustive, the computational methods for the dependable recognition of the B-cell epitopes from peptide sequences are required (El-Manzalawy *et al.*, 2008). Several methods for predicting B cell epitopes based on the physico-chemical properties of the amino acids have been developed (Saha and Raghava, 2006). The present study was performed using Chou-Fasman Beta-Turn Prediction, Emini Surface Accessibility Prediction, Karplus and Schulz Flexibility Prediction, Kolaskar and Tongaonkar Antigenicity, Parker Hydrophilicity Prediction, and Linear Epitope Prediction. Beta-turn, surface accessible, antigenic, flexible, hydrophilic, and linear epitope regions are distributed in the general region of the Kor-ApxIVA. A high value is shown at the peptide position from 568 to 636 in the beta-turn and flexibility prediction analysis, and the surface accessibility and hydrophilicity. Two epitopes of ApxIVA were found through the generation of monoclonal antibodies between 1 and 866 amino acids (Huang *et al.*, 2006). ApxIV also has three more epitopes-one between 867 and 1022 amino acids and two between 1023 and 1863 amino acids (Huang *et al.*, 2006). ApxIV that contained amino acids from 418 to 645 induces immunity and protection against *A. pleuropneumoniae* (Wang *et al.*, 2009).

In the present study, the Kor-ApxIVA showed a high rate of homology compared with the other accession numbers of ApxIVA, and was predicted for linear B-cell epitopes and conserved domains with determined peptide sequences. This genetic analysis of the Kor-ApxIVA is thought to be an important foundation for future biological and functional research on ApxIVA.

Acknowledgements

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