Rev Argent Microbiol. 2018;50(1):12–22

asociación argentina de microbiología

REVISTA ARGENTINA DE MICROBIOLOGÍA

www.elsevier.es/ram

ORIGINAL ARTICLE

Characterization of *Actinobacillus pleuropneumoniae* field strains antigenically related to the 3-6-8-15 group from diseased pigs in Japan and Argentina

Ho To[a,*], Kaho Teshima[a], Shinya Nagai[a], Gustavo C. Zielinski[b,*], Tomohiro Koyama[a], Jina Lee[a], Fernando A. Bessone[b], Tetsuji Nagano[a], Atsushi Oshima[a], Nobuyuki Tsutsumi[a]

[a] *Nippon Institute for Biological Science, 9-2221-1 Shinmachi, Ome, Tokyo 198-0024, Japan*
[b] *Animal Health Group, Estacion Experimental Agropecuaria Marcos Juarez, INTA, CC n°21 (2580), Marcos Juarez, Province of Cordoba, Argentina*

Received 5 January 2017; accepted 20 April 2017
Available online 5 September 2017

KEYWORDS
Actinobacillus pleuropneumoniae;
apx;
cps;
LPS;
Serovars 8 and 15

Abstract The objectives of this study were to determine the serovar of a collection of *Actinobacillus pleuropneumoniae* strains within the 3-6-8-15 cross-reacting group and to analyze their phenotypic and genetic properties. Based on the serological tests, forty-seven field strains of *Actinobacillus pleuropneumoniae* isolated from lungs with pleuropneumonia lesions in Japan and Argentina were found to be serovars belonging to the 3-6-8-15 cross-reacting group. By using a capsule loci-based PCR, twenty-nine (96.7%) and one (3.3%) from Japan were identified as serovars 15 and 8, respectively, whereas seventeen (100%) from Argentina were identified as serovar 8. The findings suggested that serovars 8 and 15 were prevalent within the 3-6-8-15 cross-reacting group, in Argentina and Japan, respectively. Phenotypic analyses revealed that the protein patterns observed on SDS–PAGE and the lipopolysaccharide antigen detected by immunoblotting of the reference and field strains of serovars 8 and 15 were similar to each other. Genetic (*16S rDNA*, *apxIIA*, *apxIIIA*, *cps*, *cpx* genes, *apx* and *omlA* patterns) analyses revealed that the *apxIIA* and *apxIIIA* genes of the field strains of serovars 8 and 15 were similar to those of the reference strains of serovars 3, 4, 6, 8 and 15. The results obtained in the present study may be useful for the development of more effective vaccines against disease caused by *A. pleuropneumoniae* by including the homologous antigens to the most prevalent serovars in specific geographical areas.

* Corresponding authors.
E-mail addresses: toho@nibs.or.jp (H. To), zielinski.gustavo@inta.gob.ar (G.C. Zielinski).

https://doi.org/10.1016/j.ram.2017.04.010

PALABRAS CLAVE
Actinobacillus pleuropneumoniae;
apx;
cps;
LPS;
Serotipos 8 y 15

Caracterización de cepas de *Actinobacillus pleuropneumoniae* antigénicamente relacionados al grupo 3-6-8-15 obtenidos de cerdos infectados naturalmente en Japón y Argentina

Resumen Los objetivos del presente estudio fueron determinar el serovar de una colección de cepas de *Actinobacillus pleuropneumoniae* pertenecientes al grupo 3, 6, 8, 15 de reacciones cruzadas y analizar sus propiedades fenotípicas y genéticas. En base a técnicas serológicas se determinó que cuarenta y siete cepas de *A. pleuropneumoniae* aisladas a partir de pulmones con lesiones de pleuroneumonía en Japón y Argentina pertenecen al grupo 3, 6, 8, 15. Mediante el uso de PCR basado en *locus* capsulares, veintinueve (96.7%) y una (3.3%) de los aislados japoneses fueron identificados como serovar 15 y 8 respectivamente, mientras que diecisiete (100%) de los aislados argentinos resultaron pertenecer al serotipo 8. Este hallazgo sugirió que los serovares 8 y 15 fueron los prevalentes dentro del grupo 3, 6, 8, 15 en Japón y Argentina, respectivamente. El análisis fenotípico reveló que los perfiles proteicos determinados por SDS-PAGE, y de antígenos lipopolisacáridos estudiados por *inmunoblot*, de las cepas de referencia y de campo de los serovares 8 y 15 fueron similares entre sí. El análisis genético (*16S rDNA*, *apxIIA*, *apxIIA*, *cps*, genes *cpx*, *apx* y los perfiles *omlA)* reveló que los genes *apxIIA y apxIIIA* de las cepas de campo de los serovares 8 y 15 fueron similares a sus homólogos de las cepas de referencia de los serovares 3, 4, 6, 8 y 15. Los resultados obtenidos en el presente estudio pueden ser útiles para el desarrollo de vacunas más efectivas contra la enfermedad causada por *A. pleuropneumoniae*, al posibilitar incluir antígenos homólogos a los serovares prevalentes en las áreas geográficas de interés.

Introduction

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia, an economically important bacterial disease of swine[7]. The virulence of *A. pleuropneumoniae* has been linked with exotoxins, capsular polysaccharides (CPS), lipopolysaccharides (LPS) and membrane proteins[5,7]. To date, 15 serovars have been recognized mainly on the basis of the antigenic properties of CPS and the O-polysaccharide (O-PS)[3,5,24,25], and another one, serovar 16, was proposed based on serology alone[26].

It has been shown that the characterization of the *A. pleuropneumoniae* serovar involved is useful for understanding the epidemiology of an outbreak, for preparing vaccines for the control of the disease and for serological monitoring of infected herds[7]. Cross-reactions between some serovars (1, 9 and 11; 4 and 7; and 3, 6, 8 and 15) and variable results between individual batches of test sera are usually observed in conventional serotyping tests[7–9,13,14]. To overcome these limitations, PCR assays based on the capsule loci have been developed for serotyping of *A. pleuropneumoniae* strains[4,31,32].

Currently, numerous researchers have identified serovar 8 or 15 within the 3-6-8-15 cross-reacting group[3,5,9,12,16,17,21,23,31]. An understanding of the basic characteristics of strains of serovar 8 or 15 may be critical for the design of a vaccine against the infection by serovars belonging to this cross-reacting group. The aims of this work were to determine the serovars of *A. pleuropneumoniae* strains belonging to the 3-6-8-15 cross-reacting group isolated from pneumonic lesions of naturally infected dead or diseased pigs in Japan and Argentina. The serovar-identified strains were then characterized phenotypically and genetically by different techniques and compared to reference strains.

Materials and methods

Bacterial strains and culture conditions

The bacterial strains used in this study included nine reference strains of *A. pleuropneumoniae* (4074, serovar 1; CCM5870, 2; S1421, 3; M62, 4; K17, 5a; Femo, 6; 405 and CCM3803, serovar 8 and HS 143, 15), seventeen strains randomly chosen from 35 strains isolated from pneumonic naturally infected dead or diseased pigs in Argentina and 30 strains from pigs suffering from acute pleuropneumonia in Japan. The source of the field strains of *A. pleuropneumoniae* is shown in Table 1. The initial analyses revealed that these field strains possess the following toxin-related genes: *apxIBD*, *apxIICA*, *apxIIICA*, *apxIIIBD* and *apxIVA*. The characteristics of some Argentine and Japanese strains were reported in a Master's thesis by Fernando Bessone at the University of Buenos Aires, Argentina in 2012[2] and at the 4th Asian Pig Veterinary Society Congress in Tsukuba, Japan, in 2009, respectively. In addition to the reference strains, four field strains, each from Japan and Argentina, were randomly chosen for the phenotypic and genetic analyses.

Actinobacillus pleuropneumoniae strains were cultured in chocolate agar (BD, Becton, Dickinson Co., Detroit, MI, USA) or in heart infusion medium (BBL, Cockeysville, MD, USA) supplemented with 0.3% yeast extract (dried yeast

Table 1 Source and some serological characteristics of *Actinobacillus* pleuropneumoniae strains of serovars 8 and 15

Isolate (serovar)	Source	Serotyping tests	
		AGP	COA & IBA
Reference			
4074 (1)	Argentina	1	Not tested
CCM5870 (2)	Switzerland	2	Not tested
S1421 (3)	Switzerland	3	Not tested
M62 (4)	U.S.A.	4	Not tested
K17 (5a)	U.S.A.	5a	Not tested
Femo (6)	Denmark	6	Not tested
405 (8)	Ireland	8	8, 15, 3, 6
CCM3803 (8)		8	8, 15, 3, 6
HS143 (15)	Australia	15, 7	15, 8, 3, 6
Field			
ARG43 (8)	Cordoba, Argentina (August, 2005)	8, 15, 3, 6	8, 15, 3, 6
ARG45(8)	Santa Fe, Argentina (September, 2004)	8, 15, 3, 6	8, 15, 3, 6
ARG63 (8)	Cordoba, Argentina (March, 2001)	8, 15, 3, 6	8, 15, 3, 6
ARG65(8)	Cordoba, Argentina (September, 2007)	8, 15, 3, 6	8, 15, 3, 6
13 other Argentine strains (8)	Argentina (between December, 2009 and December, 2010)	8, 15, 3, 6	Not tested
NBAP008 (15)	Kanto region, Japan (October, 2003)	15, 7	15, 8, 3, 6
NBAP009 (8)	Kanto region, Japan (March, 2009)	8, 15, 3, 6	8, 15, 3, 6
FN01	Kyushu region, Japan (September, 2012)	15, 8, 3, 6	Not tested
FN40	Kyushu region, Japan (September, 2013)	15, 8, 3, 6	Not tested
26 other Japanese strains (15)	Kyushu region, Japan (between September, 2012 and September, 2013)	15, 8, 3, 6	Not tested

AGP: reactions by agar gel precipitation; COA: coagglutination assay; IBA: immunoblotting assay.

extract-S, Nippon Seiyaku, Tokyo, Japan) and 0.005% β-nicotinamide adenine dinucleotide (Oriental Yeast Tokyo, Japan). *Escherichia coli* was grown in LB medium. When appropriate, the LB medium was supplemented with ampicillin (50 μg/ml).

Serological characterization

The immunological-based serotyping was carried out by an agar gel precipitation test (AGP) and coagglutination test (COA) using rabbit antisera against serovars 3, 6, 8 and 15 of *A. pleuropneumoniae*[20].

PCR detection of the *apx* and *omlA* genes

Apx toxin gene PCR profiling (detecting the presence of *apxICA*, *apxIBD*, *apxIICA*, *apxIIICA*, *apxIIIBD* and *apxIVA*) and outer membrane lipoprotein (*omlA*) PCR typing were examined as previously described[6,10,27]. The primers used in this study are detailed in Table 3.

PCR, cloning and sequencing of *cps*, *cpx*, *16S rDNA* and *apxA* genes

The serovars of field isolates were determined using PCR based on capsule loci[4,11,18,31,32]. Capsule loci (*cps* or *cpx* gene) typing was done as four single-reaction PCRs, one each for serovars 3, 6, 8 and 15, based on existing multiplex PCRs[4,31,32]. A 919 bp DNA fragment encoding a hypervariable region of *16 rRNA* was PCR-amplified[1]. The PCR products from the *16S rRNA*, *cps* and *cpx* genes were cleaned up using ExoSAP-I (Amersham Pharmacia Biotech, Uppsala, Sweden) and sequenced. To determine the nucleotide sequence of the open reading frame (ORF) encoding *apxIIA* or *apxIIIA*, the amplified DNA fragments encoding the full-length of *apxIIA* or *apxIIIA* were inserted into plasmid pGEM-T Easy (Promega, Madison, WI) and transformed into *Escherichia coli* XL1-Blue

by electroporation[29] DNA sequences from recombinant plasmids were determined by a primer-walking procedure[29]. In addition, phylogenetic relationships were conducted by using MEGA version 6.

Extraction of LPS and CPS

Fractions containing both LPS and CPS were extracted from bacterial cells of the five reference strains (HS143, serovar 15; 405, 8; 4074, 1; CCM5870, 2 and K17, 5a) and six field strains (NBAP008 and FN01, serovar 15; NBAP009, ARG65, ARG43 and ARG45, serovar 8) by the lysozyme–phenol–water method[15].

SDS–PAGE and immunoblotting

Overnight cultures of three reference strains (HS143, serovar 15; CCM 3803 and 405 serovar 8), and six field strains (NBAP008 and FN01, serovar 15; NBAP009, ARG65, ARG43 and ARG45, serovar 8) were used to prepare whole-cell lysates for protein analysis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting were performed as described elsewhere[28,29]. The gels containing proteins were stained with Coomassie brilliant blue R250 (CBB), whereas the gels containing LPS were stained with silver as previously described[28]. Antigenic LPSs electroblotted onto a polyvinylidene difluoride (PVDF) membrane were probed with rabbit antisera against the various serovars, and with pig sera collected one week after challenge with strains HS143 or NBAP008 of serovar 15 and visualized with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins (Zymed Laboratories, Inc., San Francisco, CA, USA) or horseradish peroxidase-conjugated goat anti-swine IgG (Rockland Immunochemicals Inc., PA, U.S.A.) and a substrate solution containing 4-chloro-1-naphthol and hydrogen peroxide.

Nucleotide sequence accession numbers

The partial *16S rRNA* sequences of the Argentine strains (ARG43, ARG45, ARG63, ARG65) and Japanese strains (NBAP009 and NBAP008) were submitted to the DDBJ/GenBank/EMBL databases under accession numbers. LC032461–LC032466. The *apxIIA* and *apxIIIA* sequences of the field strains (ARG43, ARG45, ARG63, ARG65, NBAP009 and NBAP008) and the reference strains (CCM3803, S1421, M62 and HS143) were deposited into the DDBJ/GenBank/EMBL databases under accession numbers LC043181–LC034187 and LC033888–LC033894, respectively. The nucleotide sequences of 292 bp fragment of serovar 15-specific *cpx* gene and of 1106 bp fragment of serovar 8-specific *cps* gene were submitted to the GenBank databases under accession numbers LC033493 and LC032467.

Results

Serotyping by immunological-based methods

In the AGP test, these strains tested displayed a clear line of precipitation to rabbit antiserum against serovar 8 or 15 and faint lines of precipitation to antisera against serovars 3, 6, 8, 15 or 7. These strains also showed a clear reaction by the COA with rabbit antisera against the reference strains of serovars 3, 6, 8 and 15 (Table 1).

Characterization of *A. pleuropneumoniae* strains by PCR

The PCR typing system based on the *apx* genes of six reference strains (CCM5870, serovar 2; S1421, 3; M62, 4; Femo, 6; HS143, 15; 405 and CCM3803, serovar 8) and forty-seven field strains showed the presence of *apxIBD* (genes for secretion of ApxI), *apxIICA* and *apxIIICA* (genes for expression and activation of ApxII and ApxIII, respectively), *apxIIIBD* (genes for secretion of ApxIII) and *apxIVA* (gene for expression of ApxIV) (Table 2).

The results of the PCR *olmA* gene typing showed that the field strains tested produced an *omlA* III PCR product (577 bp) identical to that seen in the reference strains of serovars 3, 6 and 15, while reference strains of serovar 8 (405 and CCM3803) produced an *omlA* II PCR product (687 bp). The reaction patterns of both the PCRs for the reference and field strains are detailed in Table 2.

Serotyping by CPS-specific gene-based PCRs

The fragment of approximately 292 bp of *cpx* gene of serovar 15 was amplified from reference strain HS143 of serovar 15 and 29 Japanese strains. Whereas, the fragment of approximately 1106 bp (*cps8a-cps8B*) of *cps* gene of serovar 8 was amplified from the reference strains of serovar 8 (405 and CCM3803), 17 Argentine strains and 1 Japanese strain. Serovar-specific primers for the reference strains of serovars 2, 3, 5 and 6 PCR amplified *cps*- or *cpx*-specific targets from the 4 reference strains (Table 2).

The nucleotide sequence similarity of the 292 bp fragment amplified from strains HS143 and from the 29 Japanese strains of serovar 15 was 100%. The sequence similarity of the 1106 bp (*cps8A-cps8B*) fragment amplified from reference strains 405 and CCM3803, 5 representative field strains (ARG43, ARG45, ARG63, ARG65, NBAP009) and other 13 field strains of serovar 8 was 100%.

Sequence analysis of the *apxIIA* and *apxIIIA* genes

The sequence analysis revealed that the *apxIIA* ORF of eleven strains tested, including three reference strains (405 and CCM3803, serovar 8 and HS143, serovar 15), four Argentine strains of serovar 8 (ARG43, ARG45, ARG63 and ARG65) and four Japanese strains (NBAP008, FN01 and FN40, serovar 15 and NBAP009, serovar 8) was 2868 bp in size (encoding 956 aa) similar to that of serovars 1, 2, 3, 4, 5, 6, 7, 9, 11, 12 and 13. The amino acid sequence similarities of ApxIIA among various serovars were

Table 2 Some genetic characteristics of *Actinobacillus pleuropneumoniae* strains of serovars 8 and 15

Isolate (serovar)	*omlA* typing	Apx toxin gene PCR profiling	Positive PCR reaction for serovar
Reference			
4074 (1)	*omlA* I	*apxICA*, *apxIBD*, *apxIICA* and *apxIVA*	Not tested
CCM5870 (2)	*omlA* II	*apxIBD*, *apxIICA*, *apxIIICA*, *apxIIIBD* and *apxIVA*	2
S1421(3)	*omlA* III	*apxIICA*, *apxIIICA*, *apxIIIBD* and *apxIVA*	3
M62 (4)	*omlA* VI	*apxIBD*, *apxIICA*, *apxIIICA*, *apxIIIBD* and *apxIVA*	Not tested
K17 (5a)	*omlA* V	*apxICA*, *apxIBD*, *apxIICA* and *apxIVA*	5
Femo (6)	*omlA* III	*apxIBD*, *apxIICA*, *apxIIICA*, *apxIIIBD* and *apxIVA*	6
HS143 (15)	*omlA* III	*apxIBD*, *apxIICA*, *apxIIICA*, *apxIIIBD* and *apxIVA*	15
405(8)	*olmA* II	*apxIBD*, *apxIICA*, *apxIIICA*, *apxIIIBD* and *apxIVA*	
CCM3803 (8)			
Field			
ARG43 (8)	*omlA* III	*apxIBD*, *apxIICA*, *apxIIICA*, *apxIIIBD* and *apxIVA*	8
ARG45(8)			
ARG63 (8)			
ARG65(8)			
13 other Argentine strains (8)			
NBAP009 (8)	*omlA* III	*apxIBD*, *apxIICA*, *apxIIICA*, *apxIIIBD* and *apxIVA*	8
NBAP008 (15)	*omlA* III	*apxIBD*, *apxIICA*, *apxIIICA*, *apxIIIBD* and *apxIVA*	15
FN01			
FN40			
26 other Japanese strains (15)			

99.4–100% (ApxIIA sequences of serovars 1, 2, 3, 5, 6, 7, 9, 11, 12 and 13 were obtained from the GenBank under numbers EFM85442.1, EFL77686.1, ABY69525, ABN74052.1, EFL80296.1, ACE61660.1, EFM94174.1, EFM98503.1, EFN00640.1 and EFN02774.1, respectively). The size of *apxIIIA* ORF of the representative field strains tested of serovars 8 and 15 was 3156 bp (encoding 1052 aa), similar to that of reference strains of serovars 3, 4, 6, 8 and 15, but different from those of strains of serovar 2 (3147 bp for reference strain 1536 and 3150 bp for Danish strain 4226, encoding 1049 and 1050 aa, respectively) (Table 4).

Protein and LPS profiles

The CBB-stained SDS–PAGE patterns of whole-cell proteins of three reference strains (HS143, serovar 15; CCM 3803 and 405 serovar 8), and six field strains (NBAP008 and FN01, serovar 15; NBAP009, ARG65, ARG43 and ARG45, serovar 8) are shown in Figure 1. Six major polypeptides (approximately 96, 60, 46, 40, 32 and 23 kDa) were found to be common to the reference and field strains of serovars 8 and 15. The polypeptide patterns of *A. pleuropneumoniae* strains were similar to each other, with minor differences in the range below 14.3 kDa.

A high-molecular-mass area of long-chain lipopolysaccharide and a ladder-like pattern typical of smooth LPS of *A. pleuropneumoniae* were observed in gels stained with silver, and in the immunoblots probed with rabbit antisera raised against *A. pleuropneumoniae* strains HS143 and NBAP008 or with pig sera collected one week after challenge with strains HS143 and NBAP008. Immunoblotting

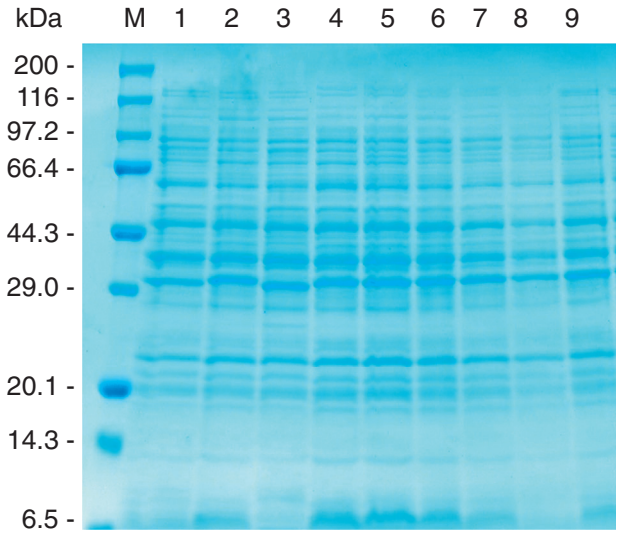

Figure 1 Whole-cell protein patterns of *A. pleuropneumoniae* strains of serovars 8 and 15 on 12.5% polyacrylamide gel stained with CBB. Lanes: 1, HS143 (serovar 15); 2, NBAP008 (serovar 15); 3, CCM 3803 (serovar 8); 4, FN01 (serovar 15); 5, NBAP009 (serovar 8): 6, ARG65 (serovar 8); 7, ARG43 (serovar 8); 8, ARG45 (serovar 8); and 9, 405 (serovar 8). M, molecular weight marker in kilodaltons (kDa).

analysis revealed that LPS of the reference and field strains (HS143 and NBAP008) of serovar 15, and of the reference (405) and field strains (ARG43, ARG45, ARG63, ARG65, NBAP009) of serovar 8 reacted strongly not only with rabbit

Table 3 Primers used in this study

Primer name	Sequence (5′ to 3′)	Target gene	Amplicon size (bp)	Reference
SSU-bact-27f	AGAGTTTGATCMTGGCTCAG	*16S rDNA*	919	1
SSU-bact-907r	CCGTCAATTCMTTTRAGTTT			
LPF	AAGGTTGATATGTCCGCACC			10
LP1R	TTTATAATGGTACCATCTTCGC	*omlA* I	809	
LP2R	TTTTACTAGAATGGTCATATTOC	*omlA* II	687	
LP3R	TATTTGGAGCTGTTTTTGTTGAT	*omlA* III	577	
		olmA IV	627	
LP4R	ACCTGCAATCTTATTAATCG	*omlA* V	418	
XICA-L	TTGCCTCGCTAGTTGCGGAT	*apxICA*	2420	6
XICA-R	TCCCAAGTTCGAATGGGCTT			
XIICA-L	CCATACGATATTGGAAGGGCAAT	*apxIICA*	2088	6
XIICA-R	TCCCCGCCATCAATAACGGT			
XIIICA-L	CCTGGTTCTACAGAAGCGAAAATC	*apxIIICA*	1755	6
XIIICA-R	TTTCGCCCTTAGTTGGATCGA			
XIBD-L	GTATCGGCGGGATTCCGT	*apxIBD*	1447	6
XIBD-R	ATCCGCATCGGCTCCCAA			
XIIIBD-L	TCCAAGCATGTCTATGGAACG	*apxIIIBD*	968	6
XIIIBD2-R	AACAGAATCAAAATCAGCTTGGTT			
APXIVA-1L	TGGCACTGACGGTGATGAT	*apxIVA*	441	27
APXIVA-1R	GGCCATCGACTCAACCAT			
AP2F	CGCAGCCGGACAAAAACAAATACACG	*cps2AB*	1400	11
AP2R	CACCCCATGAATCGACTGATTGCCAT			
AP5F	TTTATCACTATCACCGTCCACACCT	*cps5*	1140	18
AP5R	CAT TCG GGT CTT GTG GCT ACT AAA			
AP3DF	CTATCATCTGTGCAAGCTTACTACAC	*cps9D*	520	4
AP3DR	GGTTTAGAGGGGCAAATTGACTTG			
AP6NF	AACCACTCACTTTCCACATTA	*cps6D-cps6E*	718	32
AP6NR	AATCGGAAGGTTTTGGTCTCGTG			
AP8NF	TTAGTTGCGCAAACGGCTTTTGAA	*cps8A-cps8B*	1106	32
AP8NR	GATTAAACTGGTCCGTCGAAATG			
Ap15cF	GGGGATCGAAAGGCTATGG	*cpx15D*	269	31
Ap15eR	CTGCGGTAATCGCTACCATTATCC			
ApxIIA1F[a]	ATGTCAAAAATCACTTTGTCA	*apxIIA*	2868	29
ApxIIA2R	TTAAGCGGCTCTAGCTAATTG			
ApxIIIAF1[a]	ATGAGTACTTGGTCAAGCATG	*apxIIIA*	3150	29
ApxIIIAR4	TTAAGCTGCTCTAGCTAGGTT		3156	

[a] Primers for amplification of the full-length DNA fragments of *apxIIA* and *apxIIIA* were designed on the basis of the sequences of *apxIICAB* and *apxIIICAB* genes of strain CVI13261 (serovar 9, accession no. X61111) and 405 (serovar 8, accession no. X80055), respectively.

antisera against serovars 3, 6, 8, 15 (Table 1) but also with sera derived from serovar 15-infected pigs (Fig. 2).

Phylogenetic analysis

Similarity analysis of the partial *16S rRNA* sequences of the *A. pleuropneumoniae* strains showed homology ranging from 99.2 to 99.8% with those of other *A. pleuropneumonia* serovars available in the GenBank database.

In the phylogenetic analysis of a 919 bp hypervariable region of *16S rRNA* gene, a group of the field strains of serovar 8 clustered closest to a group of strains of serovar 15 (Fig. 3). In contrast, serovar 8 reference strains (405 and CCM3803) and Danish field strain 11498 clustered in a different group including serovars 3, 5, 6, 7, 12, 13 and 14.

Discussion

The forty-seven field strains (including 30 Japanese strains and 17 Argentine strains) were considered to belong to the 3-6-8-15 cross-reacting group by the immunological-based method. The capsule loci-based typing revealed that eighteen and twenty-nine strains were serovars 8 and 15, respectively. By traditional serological methods, the final identification of the serovars within the 3-6-8-15 cross-reacting group is extremely difficult[5,7–9,13,23]. It has been known that cross-reactions between serovars 3, 6, 8 and 15 could be due to common epitopes located on the LPS O-chains[5,8,9,24,25]. Our observation that conventional serotyping tests in combination with serovar-specific PCR could identify the actual serovar of isolates within 3-6-8-15 cross-reacting group is consistent with data from previous reports[9,17,23]. The finding concerning the serological characterization results

Table 4 Deduced amino acid sequence similarities among ApxIIIA from 17 *Actinobacillus pleuropneumoniae* strains

Strain (serovar) of ApxIII	% Amino acid similarity with ApxIIIA from various strains[a]																
	4226	1536	JL03	S1421	M62	Femo	405	CCM 3803	HS 143	NBAP 008	FN 01	FN 40	ARG 45	ARG 63	NBAP 009	ARG 43	ARG 65
4226 (2)[b]	100																
1536 (2)	99.05	100															
JL03 (3)	**96.29**	**95.52**	100														
S1421 (3)[c]	**96.38**	**95.61**	99.90	100													
M62 (4)	**96.18**	**95.32**	99.90	100	100												
Femo (6)	**96.38**	**95.61**	99.90	100	100	100											
405 (8)	**96.38**	**95.61**	99.90	100	100	100	100										
CCM3803 (8)	**96.38**	**95.61**	99.90	100	100	100	100	100									
HS143 (15)	**96.38**	**95.61**	99.90	100	100	100	100	100	100								
NBAP008 (15)	**96.38**	**95.61**	99.90	100	100	100	100	100	100	100							
FN01(15)	**96.38**	**95.61**	99.90	100	100	100	100	100	100	100	100						
FH40 (15)	**96.38**	**95.61**	99.90	100	100	100	100	100	100	100	100	100					
ARG45 (8)	**96.38**	**95.61**	99.90	100	100	100	100	100	100	100	96.38	96.38	100				
ARG63 (8)	**96.38**	**95.61**	99.90	100	100	100	100	100	100	100	100	100	100	100			
NBAP009 (8)	**96.19**	**95.42**	99.71	99.81	99.81	99.81	99.81	99.81	99.81	99.81	99.81	99.81	99.81	99.81	100		
ARG43 (8)	**96.29**	**95.52**	99.81	99.81	99.90	99.90	99.90	99.90	99.90	99.90	99.90	99.90	99.90	99.90	99.90	100	
ARG65 (8)	**96.29**	**95.52**	99.81	99.90	99.90	99.90	99.90	99.90	99.90	99.90	99.90	99.90	99.90	99.90	99.71	99.81	100

[a] Values showing <99.00% amino acid sequence similarity are highlighted in bold.

[b] The *ApxIIIA* sequences of strains Femo, 4662, 1536, JL03 and 405 were obtained from the GenBank databases under accession nos. EFM91631, EFL78275, P55130, ABY69902 and X80055, respectively.

[c] The *apxIIIA* sequences of strains S1421, M62, CCM3803, HS143, NBAP008, NBAP009, ARG43, ARG45, ARG63, ARG65 have been submitted to the DDBJ/EMBL/GenBank databases.

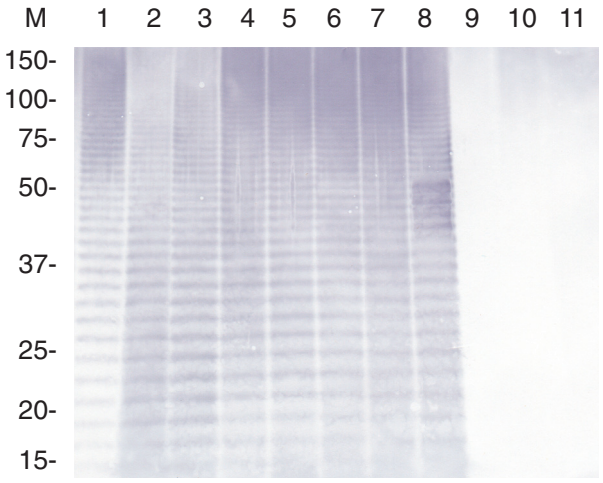

Figure 2 Immunoblot analysis of the purified LPS of *A. pleuropneumoniae* strains of serovars 8 and 15 probed with sera derived from pigs experimentally infected with reference strain HS143 of serovar 15. Lanes: 1, HS143 (serovar 15); 2, 405 (serovar 8); 3, NBAP008 (serovar 15); 4, FN01 (serovar 15); 5, NBAP009 (serovar 8): 6, ARG65 (serovar 8); 7, ARG43 (serovar 8); 8, ARG45 (serovar 8); 9, 4074 (serovar 1); 10, CCM5870 (serovar 2); and 11, K17 (serovar 5a). M, molecular weight marker in kilodaltons.

underlines the significance of capsule loci-based PCR when the immunological-based serotyping seems questionable.

It is also worth noting that the sequence analysis of the *cpx* fragment from strain HS143 and 29 field strains of serovar 15 revealed that the size of the PCR product was 292 bp instead of 269 bp as reported by Turni et al.[31] The nucleotide sequence analyses of the 292 bp fragments amplified from strains HS143 and from the 29 Japanese *A. pleuropneumoniae* strains of serovar 15 support the results mentioned on the size of the PCR product of serovar 15 presented in this study.

The serovar prevalence among strains recovered from diseased animals was reported to vary depending on the continent and time[5,8]. In a review of seroprevalence around the world, 25 of 27 countries reported the presence of serovars 3, 6 or 8[5]. Updating epidemiological data is thought to be important since the distribution of serovars within a country may change over time. For example, serovars 5 and 7 currently dominate in Canada[9], whereas until the 1990s, it was serovar 1[8]. Recently, several investigations showed that serovars 8 and 15 are highly prevalent among diseased pigs in UK[17,23] and Australia[3,31], respectively. In addition, serovar 8 was also found to be the predominant serovar within the 3-6-8-15 cross-reacting group in North America[8,9]. Similar to what has been reported in North America, the current study found that serovars

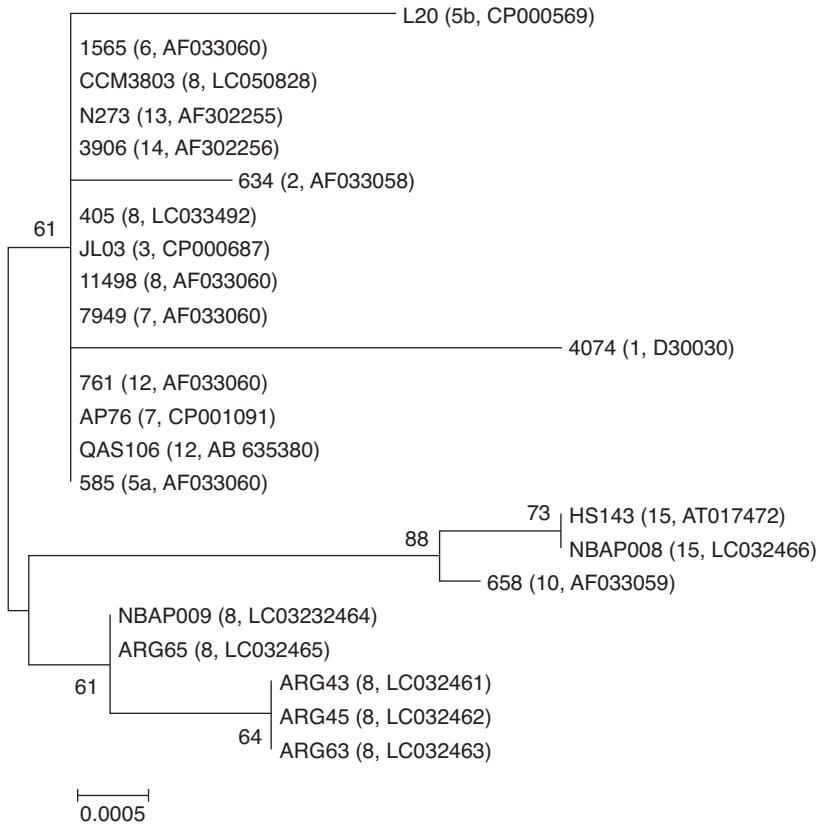

Figure 3 Neighbor-joining phylogenetic tree based on the partial nucleotide sequences of *16S rRNA* gene of 23 *A. pleuropneumoniae* strains (serovars and accession numbers are given in brackets). The tree was constructed by using MEGA version 6. Bootstrap values of 500 simulations are shown at the branches. The field strains mentioned in the present study are shown in bold. The bar with a number indicates genetic distance.

8 and 15 were prevalent serovars within the 3-6-8-15 cross-reacting group in various parts of Argentina and Japan, respectively. In Argentina, a prevalence study of *A. pleuropneumoniae* clinical isolates collected from diseased pigs in 1988[5] by an immunological-based method (COA) showed that serovar 3 was the predominant serovar within the 3-6-8-15 cross-reacting group. The presence of *A. pleuropneumoniae* isolates of serovar 8 in Argentina was found in 1996[33], and more recently, an investigation of *A. pleuropneumoniae* clinical isolates[2] found 39% of isolates from a total collection of 131 Argentine strains belonging to the 3-6-8-15 group. Our results for the prevalence of serovars within the 3-6-8-15 cross-reacting group in Argentina were different from those reported previously[5]. The reasons for the discrepancy between their results and ours are not clear but may reflect variations in the course of time studied and serotyping method used, the immunological-based method (COA) versus the serovar-specific PCR method (based on CPS-specific genes). In addition, serovar switching over time has also been reported in several countries[8]. This is what could have happened in Argentina because of changes in swine genetics and production system. Our observation that serovar 15 is the predominant serovar within the 3-6-8-15 cross-reacting group, isolated from diseased pigs in Japan is consistent with data from previous reports by Morioka et al.[21] and Ito[12].

The toxin gene profile for the thirty Japanese and seventeen Argentine strains was uniform and showed the presence of *apxIBD*, *apxIICA*, *apxIIICA*, *apxIIIBD* and *apxIVA*, suggesting that the toxin gene pattern of these strains was typical of *A. pleuropneumoniae* serovars 2, 4, 6, 8 and 15[3], because strains of serovar 3 lack *apxIBD* genes[6].

The results showed that the *omlA* genes of the field strains of serovar 8 from Argentina and Japan are different from those of the reference strains. Data from a previous report[10] showed that the *omlA* genes of *A. pleuropneumoniae* strains corresponded to their serovars, with the exception of divergence in the *omlA gene* between the reference strains and the Danish field strains of serovar 8. The present finding concerning divergence of the *omlA* gene of serovar 8 supports the previously reported results by Gram et al.[10]

The results of the PCR *apx* gene typing and the PCR *omlA* gene typing suggested that the field strains of *A. pleuropneumoniae* from Japan and Argentina belonged to serovars 6, 8 and 15.

The phylogenetic analysis based on a 919 bp hypervariable region of the *16S rRNA* gene found that the field strains of serovar 8 are genetically similar to each other but different from the reference strains of serovar 8. The reasons for this difference are not known but may be due to differences in the geographical origin of the strains or differences between the recently isolated strains and the older ones, which were isolated more than twenty years ago.

The sequence analyses revealed that the *apxIIA* genes of the field and reference strains of serovars 8 and 15 were similar to those of the reference strains of the other serovars. Whereas the *apxIIIA* genes of the field strains of serovars 8 and 15 were similar to those of the reference strains of serovars 3, 4, 6, 8 and 15, suggesting that the *apxIIIA* gene is more preserved than the *omlA* gene of strains belonging to the 3-6-8-15 cross-reacting group.

The whole-cell protein patterns of the three reference strains (405, CCM 3803 and HS143), three Argentine strains (ARG43, AGR 45 and ARG 65) and three Japanese strains (NBAP009, NBAP008 and FN01) of serovars 8 and 15 were similar to each other by one-dimension SDS–PAGE, which is consistent with data from previous reports[19,22]. Those investigators found that the whole-cell protein patterns of *A. pleuropneumoniae* serovars 1–8 are very similar to each other. In SDS–PAGE and the immunoblotting analysis, LPS of the reference and field strains of serovars 8 and 15 were found to be antigenically similar to each other. The serological typing by AGP, COA and IBA revealed cross-reactions between LPS of the *A. pleuropneumoniae* strains tested with rabbit antisera against serovars 3, 6, 8 and 15. Additionally, the results of the serological typing together with those of the immunoblotting analysis (Fig. 2) suggested that the LPS of serovars 8 and 15 was antigenically different from those of serovars 1, 2 and 5, which was supported by data from previous reports[5,7,9,24,25]. The sequence analysis revealed that the *apx* genes of the field strains of serovars 8 and 15 were almost identical to those of the reference strains of serovars 3, 4, 6, 8 and 15, but different from those of serovar 2 at the carboxyl terminus, supporting results previously reported[29]. That investigation[29] revealed that (i) the ApxIIA proteins of serovars 2, 3, 4, 6, 8 and 15 are genetically and antigenically very similar to those of the other *A. pleuropneumoniae* serovars and (ii) the apxIIIA proteins have two variants, one in strains of serovar 2 and the other in strains of serovars 3, 4, 6, 8 and 15.

In conclusion, the results of the present study revealed that (i) serovars 8 and 15 are prevalent ones within the 3-6-8-15 cross-reacting group in Argentina and Japan, respectively; and (ii) the lipopolysaccharide antigen and Apx toxins of the field strains of serovars 8 and 15 are similar to those of the reference strains of serovars 8 and 15. Commercially toxin-type vaccines have been shown to give high protection against a wide variety of *A. pleuropneumoniae* serovars[7,30]. However, a recent trial with one of these vaccines showed that the vaccine was unable to provide significant protection against strain HS143 (serovar 15) challenge[30]. Therefore, the present findings concerning antigenic similarities of LPS and Apx toxins of serovars 8 and 15 should be taken into consideration when designing vaccines for countries where isolates belonging to serovars 8 and/or 15 have been frequently found.

Authors' contributions

FAB, HT, JL, KT and TK conducted the processing and analysis of samples in the laboratory, analyzed the data, and helped to draft the manuscript. AO, NT, SN and TN contributed to manuscript writing. HT and GCZ contributed to the design of the study and manuscript writing. All authors read and approved the final manuscript.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

The authors would like to thank Professor Pat Blackall (University of Queensland, Australia) for providing reference strain HS143, Professor Marcelo Gottschalk (University of Montreal, Canada) for providing *A. pleuropneumoniae* strains; Dr. Conny Turni (University of Queensland, Australia) and Emeritus Professor Chihiro Sasakawa (University of Tokyo) for their advice during preparation of the manuscript.

References

1. Becker K, Harmsen D, Mellmann A, Meier C, Schuumann P, Peters G, Von Eiff C. Development and evaluation of a quality-controlled ribosomal sequence database for 16S ribosomal DNA-based identification of *Staphylococcus* species. J Clin Microbiol. 2004;42:4988–95.
2. Bessone FA, Tesis de Magister en Salud Animal Estudio epidemiológico molecular de *Actinobacillus pleuropneumoniae*, agente de la pleuroneumonía infecciosa porcina. Argentina: Universidad de Buenos Aires; 2012.
3. Blackall P, Klaasen H, Van Den Bosch H, Kuhnert P, Frey J. Proposal of a new serovar of *Actinobacillus pleuropneumoniae*: serovar 15. Vet Microbiol. 2002;84:47–52.
4. Bossé J, Li Y, Angen O, Weinert L, Chaudhuri R, Holden M, Williamson S, Maskell D, Tucker A, Wren B, Rycroft A, Langford P. Multiplex PCR assay for unequivocal differentiation of *Actinobacillus pleuropneumoniae* serovars 1 to 3, 5 to 8, 10, and 12. J Clin Microbiol. 2014;52:2380–5.
5. Dubreuil J, Jacques M, Mittal K, Gottschalk M. *Actinobacillus pleuropneumoniae* surface polysaccharides: their role in diagnosis and immunogenicity. Anim Health Res Rev. 2000;1:73–93.
6. Frey J, Beck M, van den Bosch J, Segers R, Nicolet J. Development of an efficient PCR method for toxin typing of *Actinobacillus pleuropneumoniae strains*. Mol Cell Probes. 1995;9:277–82.
7. Gottschalk M. Actinobacillosis. In: Zimmerman JJ, Karriker L, Ramirez A, Schvartz KJ, Stevenson GW, editors. Diseases of swine. Hoboken, NJ: Wiley; 2012. p. 653–69.
8. Gottschalk M. The challenge of detecting herds subclinically infected with *Actinobacillus pleuropneumoniae*. Vet J. 2015;206:30–8.
9. Gottschalk M, Lacouture S. *Actinobacillus pleuropneumoniae* serotypes 3, 6, 8 and 15 isolated from diseased pigs in North America. Vet Rec. 2014;174:452–3.
10. Gram T, Ahrens P, Andreasen M, Nielsen J. An *Actinobacillus pleuropneumoniae* PCR typing system based on the *apx* and *omlA* genes – evaluation of isolates from lungs and tonsils of pigs. Vet Microbiol. 2000;75:43–7.
11. Hüssy D, Schlatter Y, Miserez R, Inzana T, Frey J. PCR-based identification of serotype 2 isolates of *Actinobacillus pleuropneumoniae* biovars I and II. Vet Microbiol. 2004;99:307–10.
12. Ito H. Recent topics of serotypes/serotyping and host specificity of *Actinobacillus pleuropneumoniae*. Proc Jpn Pig Vet Soc. 2013;61:14–21 (in Japanese).
13. Ito H, Matsumoto A. Isolation and genetic characterization of an *Actinobacillus pleuropneumoniae* serovar K12:O3 strain. J Vet Diagn Invest. 2015;27:102–6.
14. Ito H, Katsuragi K, Akama S, Yuzawa H. Isolation of atypical genotype *Actinobacillus pleuropneumoniae* serotype 6 in Japan. J Vet Med Sci. 2014;76:601–4.
15. Jolie R, Mulks M, Thacker B. Antigenic differences within *Actinobacillus pleuropneumoniae* serotype 1. Vet Microbiol. 1994;38:329–49.
16. Koyama T, To H, Nagai S. Isolation of *Actinobacillus pleuropneumoniae* serovar 15-like strain from a field case of porcine pleuropneumonia in Japan. J Vet Med Sci. 2007;69:961–4.
17. Li Y, Bossé J, Williamson S, Maskell D, Tucker A, Wren B, Rycroft A, Kroll J, Hartley H, Langford P. *Actinobacillus pleuropneumoniae* serovar 8 predominates in England and Wales. Vet Rec. 2016;179:276–7.
18. Lo TM, Ward CK, Inzana TJ. Detection and identification of *Actinobacillus pleuropneumoniae* serotype 5 by multiplex PCR. J Clin Microbiol. 1998;36:1704–10.
19. Macinnes J, Rosendal S. Analysis of major antigens of *Haemophillus* (*Actinobacillus*) *pleuropneumoniae* and related organisms. Infect Immun. 1987;55:1626–34.
20. Mittal K, Higgins R, Lariviere S. Quantitation of serotype-specific and cross-reacting group-specific antigens by coagglutination and immunodiffusion tests for differentiating *Actinobacillus* (*Haemophilus*) *pleuropneumoniae* strains belonging to cross-reacting serotypes 3, 6, and 8. J Clin Microbiol. 1988;26:985–9.
21. Morioka A, Asai T, Nitta H, Yamamoto K, Ogikubo Y, Takahashi T, Suzuki S. Recent trends in antimicrobial susceptibility and the presence of the tetracycline resistance gene in *Actinobacillus pleuropneumoniae* isolates in Japan. J Vet Med Sci. 2008;70:1261–4.
22. Nicolet J, Paroz P, Krawinkler M. Polyacrylamide gel electrophoresis of whole-cell proteins of porcine strains of *Haemophilus*. Int J Syst Bacteriol. 1980;30:69–76.
23. O'Neill C, Jones S, Bossé J, Watson C, Williamson S, Rycroft A, Kroll J, Hartley H, Langford P. Prevalence of *Actinobacillus pleuropneumoniae* serovars in England and Wales. Vet Rec. 2010;167:661–2.
24. Perry M, Altman E, Brisson J, Beynon L, Richards J. Structural characteristics of the antigenic capsular polysaccharides and lipopolysaccharides involved in the serological classification of *Actinobacillus* (*Haemophillus*) *pleuropneumoniae* strains. Serodiagn Immunother Infect Dis. 1990;4:299–308.
25. Perry M, MacLean L, Vinogradov E. Structural characterization of the antigenic capsular polysaccharide and lipopolysaccharide O-chain produced by *Actinobacillus pleuropneumoniae* serotype 15. Biochem Cell Biol. 2005;83:61–9.
26. Sárközi R, Markai L, Fodor L. Identification of a proposed new serovar of *Actinobacillus pleuropneumoniae*: serovar 16. Acta Vet Hung. 2015;63:444–50.
27. Schaller A, Kuhn E, Kuhnert P, Nicolet J, Anderson T, Maclnnes J, Segers R, Frey J. Characterization of *apxIVA*, a new RTX determinant of *Actinobacillus pleuropneumoniae*. Microbiology. 1990;145:2105–116.
28. To H, Hotta A, Yamaguchi T, Fukushi H, Hirai K. Antigenic characteristics of the lipopolysaccharides of *Coxiella burnetii* isolates. J Vet Med Sci. 1998;60:267–70.
29. To H, Nagai S, Iwata A, Koyama T, Oshima A, Tsutsumi N. Genetic and antigenic characteristics of ApxIIA and ApxIIIA from *Acti-*

nobacillus pleuropneumoniae serovars 2, 3, 4, 6, 8 and 15. Microbiol Immunol. 2016;60:447–58.
30. Tumamao J, Bowles R, Van den Bosch H, Klaasen H, Fenwick B, Storie G, Blackall P. Comparison of the efficacy of a subunit and a live streptomycin-dependent porcine pleuropneumonia vaccine. Aust Vet J. 2004;82:370–4.
31. Turni C, Singh R, Schembri M, Blackall P. Evaluation of a multiplex PCR to identify and serotype *Actinobacillus pleuropneumoniae* serovars 1, 5, 7, 12 and 15. Lett Appl Microbiol. 2014;59:362–9.
32. Zhou L, Jones S, Angen O, Bosse J, Nash J, Frey J, Zhou R, Chen H, Kroll J, Rycroft A, Langford P. Multiplex PCR that can distinguish between immunologically cross-reactive serovars 3, 6, and 8 *Actinobacillus pleuropneumoniae* strains. J Clin Microbiol. 2008;46:800–3.
33. Zielinski G, Piscitelli H, Blackall P. *Actinobacillus pleuropneumoniae* serotipo 8: un nuevo serovar para Argentina. Therios. 1996;25:22–7.