The Expression of apxIVA Gene of *Actinobacillus pleuropneumoniae* Serotype 5 and Establishment of an Indirect ELISA for Distinguishing Infection and Immunization

Nianli Zou[1], Yulan Lai[1], Min Li[1], Sanjie Cao[1,2], Xintian Wen[1,2] and Yong Huang[*,1,2]

[1]*College of Veterinary Medicine, Sichuan Agricultural University. Ya'an 625014, China*

[2]*Key laboratory of Animal Disease and Human Health of Sichuan Province, Sichuan Agricultural University, Ya'an 625014, China*

Abstract: *Actinobacillus pleuropneumoniae* is the etiological agent of Porcine pleuropneumonia, which causes severe losses in pig farming. To establish an serological method to detect *A. pleuropneumoniae* infection, bioinformatics method was utilized to analyzed the sequence of *apxIVA* gene of *A. pleuropneumoniae* serotype 5 completed by our lab, a 1152 bp fragment of the N'-terminal of *apxIVA* gene was amplified and cloned into the prokaryotic expression vector pET-32a(+), a recombinant protein about 62KD was expressed upon isopropy 1-β-D- thiogalactoside (IPTG) induction. The protein could react specifically with antiserum from live *A. pleuropneumoniae* infection in western-blot. After further purification by Ni-NTA, this protein was used to establish an indirect ELISA to detect A. *pleuropneumoniae* infection. This method showed high specificity and could react positively with antibodies of live *A. pleuropneumoniae* infection while negatively with those of inactivated *A. pleuropneumoniae* immunization. In conclusion, this indirect ELISA could be used to detect A. *pleuropneumoniae* infection in mice model.

Keywords: *Actinobacillus pleuropneumoniae*, *ApxIVA*, serotype 5, indirect ELISA, infection, immunization.

Porcine pleuropneumonia is an acute or chronic respiratory disease characterized by hemorrhagic, fibrinous, and necrotic lung lesions, and *A. pleuropneumoniae* is the etiological agent [1]. The disease is highly contagious and associated with all ages of growing pigs, often co-infecting with other pathogens such as Hog cholera virus, Porcine reproductive and respiratory syndrome virus and Pasteurella multocida etc, resulting in severe economic losses in swine industry. The morbidity ranges from 8.5% to 100% while the fatality ranges from 0.4% to 100%. *A. pleuropneumoniae* infection is more prevalent under intensive breeding conditions. To date, 15 serotypes have been reported with serovars 1, 7, and 12 making up approximately 90% of isolates [2, 3].

A. pleuropneumoniae-RTX toxins (apx) is the main virulence factor and protective antigen of *A. pleuropneumoniae*. To update, four kinds of apx toxins were found produced by *A. pleuropneumoniae,* named apx I, apx II, apxIII and apxIV respectively [4]. The apx toxins produced by *A. pleuropneumoniae* varied with serotypes, but all serotypes can secrete apxIV which can be produced only during infection, but not under *in vitro* conditions [4]. ApxIV can also induce specific antibodies of high titres [5]. Based on the above character, bioinformatics method was used to analyzed the *apxIVA* gene sequence of serotype 5 completed by our lab, and a 1152 bp long fragment of the N'-terminal of *apxIVA* gene of serotype 5 was cloned and expressed to

establish an indirect ELISA for detecting *A. pleuropneumoniae* infection in mice model.

MATERIALS AND METHODOLOGY

Bacterial Strain and Grown Conditions

E. coli JM 109, DH5a and BL21 (DE3) were purchased from Invitrogen corporation (California, USA) and cultured in LB, *A. pleuropneumoniae* serotype 1 strain CVCC 259 and serotype 5 strain CVCC 263 and serotype 7 strain CVCC 265 were purchased from China institute of Veterinary Drug Control (Beijing, China) and proliferated in Tryptic Soy Broth (TSB) with 0.001% nicotinamide adenine dinucleotide (NAD).

Experiment Mice

Female Kun-Ming mice (six weeks old, 17–22 g), purchased from the experimental animal center of Sichuan University (Chengdu, China), were maintained in animal holding laboratory under controlled condition with temperature of 25±10°C, humidity of 40±10% and had free access to standard mouse diet and water.

Sera

For the production of mice antisera against live or inactivated *A. pleuropneumoniae* serotype 5, the 50% of lethal dose (LD50) were determined firstly. Six-week-old Kun-Ming mice were randomly divided into five groups, each of them consisting of eight mice. and injected intraperitoneally (i.p.) with 200 μl TSB containing *A. pleuropneumoniae* (1.0×10^8 to 1.0×10^4 CFU/mouse). The number of surviving mice was recorded 5 days after

*Address correspondence to this author at the College of Veterinary Medicine, Sichuan Agricultural University. Ya'an 625014, Sichuan, China; Tel: +868352886117; Fax: +868352886117; E-mail: hyong601@163.com

infection. LD50 was calculated by the method of Reed and Muench and determined to be 6.3×10^6 CFU. Then Six-week-old Kun-Ming mice were randomly divided into two groups, each of which consisting of twelve mice. Mice in group A were injected i.p. with 200 μl of *A. pleuropneumoniae* preparation containing 6×10^5 CFU per mouse on days 0, 14 and 28 respectively; while mice in group B were inoculated i.p. with the same amount of inactivated *A. pleuropneumoniae* as group A per mouse on days 0, 14 and 28 respectively. Mice were bled from eyes 10 days following the final boost and sera were collected. For the production of mice antisera against live *A. pleuropneumoniae* serotype 1 or 7, twelve mice were inoculated with live *A. pleuropneumoniae* preparation containing 1/10 LD50 per mouse following the method above.

Construction of Recombinant Prokaryotic Expression Vector

A. pleuropneumoniae present in 1 ml of an overnight culture were collected by centrifugation and DNA was extracted using a sodium dodecyl sulfate (SDS) - proteinase K and phenol/chloroform protocol as described in previous research [6]. Then, bioinformatics method was used to analyze the *apxIVA* gene sequence of A. pleuropneumoniae serotype 5 completed by our lab (Genbank NO: GQ332268), a pair of specific primers was used to amplify the 1152 bp fragment of the 5'-terminal of apxIV A gene. The two primers were 5'CCA <u>GAATTC</u> ACG TTA GAC GAA ACA AGG- 3' with *EcoRI* site (underlined) comprising position 655–672 of apxIVA coding sequence and 5'CTC <u>CTCGAG</u> TTA ATT CAG ATC GGA GGC A -3' with *XholI* site (underlined) comprising position 1791–1806 of apxIVA coding sequence. The oligonucleotides were synthesized by Takara biotechnology (Dalian, China) co., ltd. Amplification of the apxⅣA gene was performed with polymerase chain reaction (PCR) and the PCR products were cloned into the pMD18-T plasmid (Takara) to yield recombinant plasmid. Confirmation of clones containing recombinant plasmid was achieved by PCR and restriction enzyme (RE) digestion, and the correct clones were sequenced by Takara biotechnology (Dalian). The recombinant plasmid were then digested with *EcoRI* and *XholI* and cloned into pET-32a(+) (Novagen, Madison, USA) digested with *EcoRI* and *XholI* to generate recombinant prokaryotic expression plasmid.

Expression and Purification of *apxIVA*

The BL21 strains containing the recombinant prokaryotic plasmid were grown overnight with shaking at 37°C. The overnight cultures were diluted in the proportion of 1:100 and incubated at 37°C with vigorous shaking for 3-4 hours (h.). The production of poly-His-tailed ApxIV a proteins were induced by addition of 1 mM IPTG and incubation for a further 3-4 h. The cells were harvested and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Optimization of expression conditions at different IPTG concentration ranging from 0, 0.2, 0.4, 0.6, 0.8, 1.0 to 1.2 mM and different induction time ranging from 0, 2, 3,4, 5 to 6 h were also conducted.

For purification of recombinant *apxIVA*, the cells were harvested from liquid cultures by centrifugation and suspended in phosphate-buffered saline (PBS) (pH 7.2) containing lysozyme (1mg/ml) by 1/10 (v/v), after ice bathing for 30 min, the suspension were sonicated and centrifuged at 12000g for 10 min. The pellets were homogenated and washed with washing buffer [50 mM Tris-Hcl (PH8.0), 1 mM EDTA, 0.2% TritonX-100, 2 M urea] for three times at 10min/times and centrifugated at 12000g for 10 min. The pellets were dissolved by denaturation buffer [50 mM Tris-Hcl (PH8.0), 2 mM 2-mercaptoethanol, 8 M urea] and supernatant were collected after centrifugation. The supernatant was treated with renaturation buffer [50 mM Tris-Hcl (PH8.0), 0.1 mM oxidized glutathione, 1 mM reduced glutathione, 0.5 M urea] for overnight at 4°C and filtrated through 0.45 μM filtration membrane. Then the solution was purified on a column packed with Ni-NTA His·Bind superflow according to the manufacture's instruction (Merck, Darmstadt, Germany). The eluate from each imidazole concentration were collected and analyzed by SDS-PAGE.

Western Blot Analysis

Purified proteins were separated by SDS-PAGE, then the proteins were transferred to nitrocellulose membrane with 0.45μm pore size (Millipore Corp., USA) at 15V for 1.5 h with a Bio-Rad Transblot Cell as described in the manufacturer's specifications. The membrane was then blocked for 90 min with milk buffer (20mM Tris–HCl pH 8.0, 150mM NaCl, 0.05% Tween 20, 5% skimmed dry milk) at 37°C, and then washed with Tris-buffered saline with Tween 20 (TBST) buffer (20mM Tris-HCl PH 8.0, 150mM NaCl, 0.05 Tween-20) for three times and incubated with mouse antiserum diluted 1:100 in 0.5% bovine serum albumin (BSA) /PBS for 60 min at 37°C. The membrane was washed with TBST, then incubated with horseradish peroxidase (HRP) -labeled sheep-anti-mouse IgG (zhongshan Goldenbridge Biotechnology Co., LTD, Beijing, China) for 60 min at 37°C. Target proteins were visualized using 3,3'- diaminobenzidine (Tiangen, Beijing, China).

Development of an *apxIVA*-ELISA

A 96-well microtiter plate (Costar, USA) was coated with 100 μl of purified *apxⅣA* and incubated at 4°C overnight. The plate was then blocked for 90 min with milk buffer (5% skimmed dry milk in PBS) at 37°C and washed twice with PBS. Subsequently, 100 μl of mice sera were added and incubated at 37°C for 60 min. The samples were washed, and then incubated for 45 min with 100 μl of anti-mouse IgG diluted 1:10000 in 0.1% BSA/PBS at 37°C, washed again, and detected with 100 μl of 3,3',5,5' - tetramethyl benzidine (TMB) for 20 min at room temprature (RT). The reaction was then stopped by the addition of 35 μl of 2 M H_2SO_4. The optical density (OD) value was read at 450 nm, using a Bio-Rad model 860 plate reader (Bio-Rad, CA, USA).

Checker board method was used to determine the optimal antigen coating concentration, serum dilutions and HRP labeled rabbit anti-mouse IgG dilutions. For determine the cut-off value, twenty-four sera samples from mice uninfected with *A. pleuropneumoniae* were used as negative sera, the cut-off value was calculated using the formula: mean of the negative serum values plus three standard deviations (SDs). To determine the specificity of this method, rabbit antisera of HCV, PRRSV and PM (obtained from Dr. Qigui Yan, Sichuan Agricultural University, China) were detected

according to the method above, except that the second antibody was anti-rabbit IgG (Zhongshan, Beijing, China) diluted 1:10000 in 0.1% BSA/PBS. Finally, the method was used to detect the sera of inactivated *A. pleuropneumoniae* immunized and live *A. pleuropneumoniae* infected mice.

RESULTS

Construction of an *apxIVA* Prokaryotic Expression Vector

The *apxIVA* gene was isolated from *A. pleuropneumoniae* ATCC 263 by PCR as described in Materials and Methods. The oligonucleotides used in the PCR reaction were designed to allow the 1152 bp long fragment in 5' terminal part of *apxIVA* gene to be cloned into pET-32a(+) in frame with the poly-His purification signal. PCR and RE analysis showed that prokaryotic expression plasmid PET32a-apxIVA was successfully constructed. (Fig. **1**).
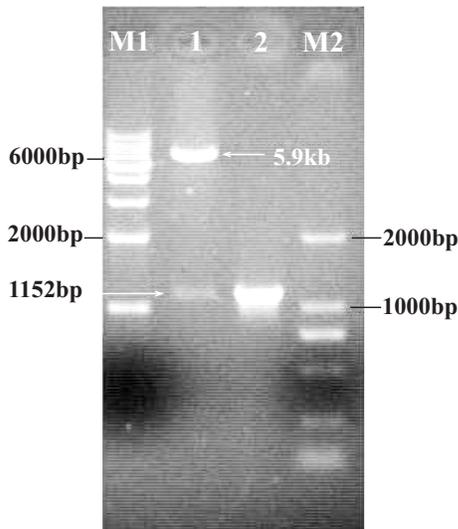

Fig. (1). Results of construction of an ApxIVa prokaryotic expression vector. M1: DNA Marker DL10000; Lane 1: prokaryotic expression vector digested with EcoR I and Xho I; Lane 2: PCR products of ApxIVa gene; M2 : DNA Marker DL2000

Prokaryotic Expression and Purification of *apxIVA*

Prokaryotic expression vector PET32a-apxIVA was induced by addition of IPTG to produce recombinant *apxIVA* toxin at different time ranged from 0, 2, 3,4, 5 to 6 h and at different IPTG concentration ranged from 0, 0.2, 0.4, 0.6, 0.8, 1.0 to 1.2 mmol L^{-1}, the results showed that a protein about 62Kd was expressed (Fig. **2**) since 1h and stabilized on 4 h post culturation at a most appropriate IPTG concentration of 0.6 mM (data not show). The recombinant *apxIVA* was expressed mainly in the form of inclusion body and Ni-NTA superflow was used to purify this his-tagged *apxIVA*. For the purified protein, a single objective band was detected by SDS-PAGE (Fig. **2**).

Western Blot Assay

To confirm the reactivity of his-tagged *apxIVA* with antibodies from *A. pleuropneumoniae* infection, the purified protein was exposed to a Western blot assay by using serum from *A. pleuropneumoniae*. The result showed that the fusion protein can react positively with serum from live *A. pleuropneumoniae* infection (Fig. **2**), while negatively with serum from inactivated *A. pleuropneumoniae* immunization.(data not show).
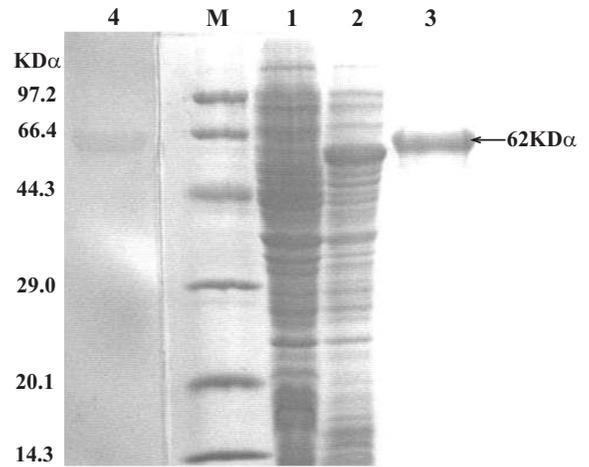

Fig. (2). Results of expression and purification and western-blot of recombinant protein. Lane M: protein marker; Lane 1: uninduced BL21 containing recombinant plasmid; Lane2: bacteria induced for 4h; Lane 3: purified Apx IVA; lane 4: western-blot result of purified recombinant protein with live *A. pleuropneumoniae* infected antisera

Indirect ELISA Based on Recombinant *ApxIVA*

The results of checker board experiment showed that a concentration of 1.0 μg/ml of apxIVA-His peptide and 1:40 serum produced the best resolution of the positive reference serum from the negative reference serum. Then 24 negative mice sera was selected randomly to react with *apxIVA*-His peptide, the average χ was 0.143, standard deviation(SD) was 0.028. The positive critical value was χ+3SD=0.226. Means that while the OD value of sample ≥0.226, the result was positive; while the OD value of sample <0.226, the result was negative. For the results of specificity test, positive sera of HCV, PRRSV and Pm were negative in the *apxIVA*-ELISA. indicating the specificity of this *apxIVA*-ELISA (Table **1**).

Table 1.   The Results of Specificity Test of *apxIVA*-ELISA

	Positive Sera		
	Sera Number	**PRRSV**	**HCV**	**Pm**
OD$_{450}$ of apxIVa- ELISA	1	0.151	0.083	0.105
	2	0.095	0.162	0.178
	3	0.133	0.098	0.135
	4	0.125	0.087	0.039

PRRSV: Porcine reproductive and respiratory syndrome virus; HCV: Hog cholera virus; Pm: Pasteurella multocida.

For the results of discrimination of infection and inactivated vaccine immunization, Sera of four mice immunized with inactivated *A. pleuropneumoniae*, four mice

Table 2. Detection of Antibodies Against *A. pleuropneumoniae* Infection and Inactivated *A. pleuropneumoniae* Immunization

		Serum			
	Animal Numbers	Inactivated App Serotype 5 Vaccinated Mice	live App Serotype 1 Infected Mice	Live App Serotype 5 Infected Mice	Live App Serotype 7 Infected Mice
OD$_{450}$ of apxIva-ELISA	1	0.171	1.145	1.297	1.175
	2	0.133	0.899	1.183	0.853
	3	0.201	1.013	1.215	0.933
	4	0.145	1.235	1.033	

App: A. pleuropneumoniae.

infected with live serotype 1, four mice infected with live serotype 5 and three mice infected with live serotype 7 *A. pleuropneumoniae* were analyzed by apxIVA-ELISA. The results showed that all the sera of mice immunized with inactivated *A. pleuropneumoniae* were negative in the apxIVA-ELISA, while all the mice infected with live serotype 1 or 5 or 7 *A. pleuropneumoniae* gave positive results (Table **2**).

DISCUSSION

Porcine pleuropneumonia was an respiratory disease hazardous to China pig industry. The virulence of the 15 serotypes of *A. pleuropneumoniae* is mainly determined by three major RTX toxins apx I, apx II and apx III [7], which are secreted by the different serotypes in various combinations, but apxIV can be produced by all 15 serotypes only during infection, but not under *in vitro* conditions [4].

The diagnosis of *A. pleuropneumoniae* infections is important for the identification of farms that are latently infected with A. pleuropneumoniae, detection of different *A. pleuropneumoniae* serotypes, inspection of the animals on their importation into a farm, or eradication of the infection. It is done routinely by serological examination of pig herds or by bacterial agent cultivation from nasal swabs or lungs with *pleuropneumonia* lesions or by PCR. Serological examination were usually carried out by detecting antibodies provoked by Apx toxin or outer membrane protein (OMP) or lipopolysaccharides (LPS) etc. OMP are common to all *A. pleuropneumoniae* serotypes [8], while LPS are serotype-specific and used to serologically distinguish infections with different serotypes of *A. pleuropneumoniae* [9], LPS was also found to give serological cross-reaction with non-pathogenic *Actinobacillus* species [10]. PCR-based methods were suitable for the rapid detection of *A. pleuropneumoniae* [11].

Vaccination is potentially an effective tool for the control of Porcine pleuropneumonia, although natural or experimental infection generally elicits at least partially protection against reinfection with another serotype [12]. Killed whole cell bacterins were commercially used in China now, and a major drawback of vaccination, is that traditional serological tests including hemmaglutination test [13, 14] and ELISA [15], cannot distinguish immunization of killed whole cell bacteria from field exposition. As *apxIVA* can be expressed by all 15 serotypes only *in vivo* conditions, therefore, antibodies against *apxIVA* should be the target of serological differentiation between immunization with killed whole cell bacteria vaccine and infection with live *A. pleuropneumoniae*.

Although an indirect ELISA based on recombinant *apxIVA* of *A. pleuropneumoniae* serotype 1 have been reported previously [16], but indirect ELISA based on recombinant *apxIVA* of serotype 5 have not been reported now. The serotype 5 is highly pathogenic, and the sequence of *apxIVA* of serotype 5 was different from those of serotype 1 and 3 in length and homology [17]. So we develop an indirect ELISA based on recombinant *apxIVA* of serotype 5 to see whether it can also detect antibodies against *A. pleuropneumoniae* infection. After the bioinformatic analysis of *apxIVA* gene sequence of serotype 5 strain CVCC 263, it was found that the 5'-terminal of *apxIVA* gene was highly antigenic, and subsequently a 1152 bp fragment in this region was amplified and expressed to establish an indirect ELISA for detecting the antibody against th*e apxIVA* of all serotypes. The results showed that the sera from inactivated vaccine immunized mice were negative, while the sera from live *A. pleuropneumoniae* infected mice were positive. So this indirect ELISA could detect *A. pleuropneumoniae* infection in a mouse model. The validation of this apxIVA-ELISA to detect *A. pleuropneumoniae* infection in pigs will be further undertaken.

ACKNOWLEDGEMENTS

ABBREVIATIONS

BSA = Bovine serum albumin

ELISA = Enzyme-linked immunosorbent assay

HRP = Horseradish peroxidase

i.p. = Intraperitoneally

IPTG = Isopropy 1-β-D-thiogalactoside

LPS = Lipopolysaccharides

NAD = Nicotinamide adenine dinucleotide

OD = Optical density

OMP = Outer membrane protein

PBS = Phosphate-buffered saline

PCR = Polymerase Chain Reaction

RE   =   Restriction enzyme

RT   =   Room temprature

SDS-PAGE =   Sodium dodecyl sulfate polyacrylamide gel
                electrophoresis

TBST   =   Tris-Buffered Saline with Tween 20

TSB   =   Tryptic Soy Broth

REFERENCES

[1]   Bosse JT, Janson H, Sheehan BJ, *et al*. *Actinobacillus pleuropneumoniae*: pathobiology and pathogenesis of infection. Microbes Infect 2002; 4(2): 225-35.

[2]   Blackall PJ, Klaasen HL, van den Bosch H, Kuhnert P, Frey J. Proposal of a new serovar of *Actinobacillus pleuropneumoniae*: serovar 15. Vet Microbiol 2002; 84(1-2): 47-52.

[3]   Komal JP, Mittal KR. Grouping of *Actinobacillus pleuropneumoniae* strains of serotypes 1 through 12 on the basis of their virulence in mice. Vet Microbiol 1990; 25(2-3): 229-40.

[4]   Schaller A, Kuhn R, Kuhnert P, *et al*. Characterization of apxIVA, a new RTX determinant of *Actinobacillus pleuropneumoniae*. Microbiology 1999; 145 (Pt 8): 2105-16.

[5]   Wang C, Wang Y, Shao M, *et al*. Positive role for rApxIVN in the immune protection of pigs against infection by *Actinobacillus pleuropneumoniae*. Vaccine 2009; 27(42): 5816-21.

[6]   Daban M, Medrano A, Querol E. Cloning, sequencing and expression of the transferrin-binding protein 1 gene from *Actinobacillus pleuropneumoniae*. Biochem J 1996; 315 (Pt 1): 257-64.

[7]   Frey J. Virulence in *Actinobacillus pleuropneumoniae* and RTX toxins. Trends Microbiol 1995; 3(7): 257-61.

[8]   Beynon LM, Griffith DW, Richards JC, Perry MB. Characterization of the lipopolysaccharide O antigens of *Actinobacillus pleuropneumoniae* serotypes 9 and 11: antigenic relationships among serotypes 9, 11, and 1. J Bacteriol 1992; 174(16): 5324-31.

[9]   Mittal KR, Bourdon S, Berrouard M. Evaluation of counterimmunoelectrophoresis for serotyping *Actinobacillus pleuropneumoniae* isolates and detection of type-specific antigens in lungs of infected pigs. J Clin Microbiol 1993; 31(9): 2339-42.

[10]   Gottschalk M, Broes A, Mittal KR, *et al*. Non-pathogenic *Actinobacillus* isolates antigenically and biochemically similar to Actinobacillus pleuropneumoniae: a novel species? Vet Microbiol 2003; 92(1-2): 87-101.

[11]   Schaller A, Djordjevic SP, Eamens GJ, *et al*. Identification and detection of *Actinobacillus pleuropneumoniae* by PCR based on the gene apxIVA. Vet Microbiol 2001; 79(1): 47-62.

[12]   Cruijsen T, van Leengoed LA, Ham-Hoffies M, Verheijden JH. Convalescent pigs are protected completely against infection with a homologous *Actinobacillus pleuropneumoniae* strain but incompletely against a heterologous-serotype strain. Infect Immun 1995; 63(6): 2341-3.

[13]   Mittal KR, Higgins R, Lariviere S, Leblanc D. A 2-mercaptoethanol tube agglutination test for diagnosis of *Haemophilus pleuropneumoniae* infection in pigs. Am J Vet Res 1984; 45(4): 715-9.

[14]   Habrun B, Bilic V, Humski A. Comparison of ELISA and 2-META assays used in serological diagnosis of infection with *Actinobacillus pleuropneumoniae* serotypes 2 and 4-7 in breeding pigs in Croatia. Prev Vet Med 1998; 36(3): 179-86.

[15]   Grondahl-Hansen J, Barfod K, Klausen J, Andresen LO, Heegaard PM, Sorensen V. Development and evaluation of a mixed long-chain lipopolysaccharide based ELISA for serological surveillance of infection with *Actinobacillus pleuropneumonia*e serotypes 2, 6 and 12 in pig herds. Vet Microbiol 2003; 96(1): 41-51.

[16]   Dreyfus A, Schaller A, Nivollet S, *et al*. Use of recombinant ApxIV in serodiagnosis of *Actinobacillus pleuropneumoniae* infections, development and prevalidation of the ApxIV ELISA. Vet Microbiol 2004; 99(3-4): 227-38.

[17]   Foote SJ, Bosse JT, Bouevitch AB, Langford PR, Young NM, Nash JH. The complete genome sequence of *Actinobacillus pleuropneumoniae* L20 (serotype 5b). J Bacteriol 2008; 190(4): 1495-6.