



NOTE

Bacteriology

Isolation and characterization of atypical *Actinobacillus pleuropneumoniae* serovar 15 lacking the *apxIIICA* genes in Japan

Kaho TESHIMA¹⁾, Haruna HIRANO²⁾, Kazutada USHIYAMA²⁾,
Kazumoto SHIBUYA¹⁾, Shinya NAGAI¹⁾, Chihiro SASAKAWA^{1,3)} and Ho TO^{1)*}

¹⁾Nippon Institute for Biological Science, Tokyo 198-0024, Japan

²⁾Yamanashi Prefecture Eastern Livestock Hygiene Service Center, 1001-1 Karakashiwa, Ishawa, Fuefuki, Yamanashi 406-0034, Japan

³⁾Medical Mycology Research Center, Chiba University, 1-8-1 Inohana, Chiba 260-8637, Japan

J. Vet. Med. Sci.
81(3): 480–485, 2019
doi: 10.1292/jvms.18-0421

Received: 22 July 2018
Accepted: 14 January 2019
Published online in J-STAGE:
1 February 2019

ABSTRACT. Six atypical *Actinobacillus pleuropneumoniae* serovar 15 strains were isolated from pneumonic lesions of naturally infected dead pigs from the same farm in Japan. Genetic analyses of *apx* genes revealed that the atypical isolates contained the toxin-associated genes *apxIBD*, *apxIIICA*, *apxIIIBD*, and *apxIVA*, but not *apxIIICA*. Coinciding with the result of the atypical gene profile, analyses of toxin protein production revealed that these atypical isolates expressed only ApxIII but not ApxII. A mouse pathogenicity test showed that the atypical isolate tested seemed to be less virulent than the typical isolates. This is the first report describing the emergence of atypical *A. pleuropneumoniae* serovar 15, which does not produce ApxII due to the absence of *apxIIICA* genes, in Japan.

KEY WORDS: *Actinobacillus pleuropneumoniae*, *apxIIICA*, *apxIICABD*, serovar 15

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia, an economically important bacterial infection of swine [6]. Although the virulence of *A. pleuropneumoniae* is multifactorial (RTX toxins, capsular polysaccharides [CPS], lipopolysaccharides [LPS], and many iron acquisition systems), the major factor primarily responsible for the development of clinical disease and typical pleuropneumonia lesions is Apx exotoxins [3, 6, 7, 10, 12, 18]. To date, 18 serovars have been recognized which variously produce four RTX (repeats-in-toxin) toxins (ApxI, ApxII, ApxIII and ApxIV) [2, 5, 21, 22]. ApxI is strongly hemolytic and cytotoxic, ApxII is weakly hemolytic and cytotoxic, ApxIII is strongly cytotoxic to porcine neutrophils and pulmonary alveolar macrophages, and ApxIV is expressed only *in vivo* and is specific to *A. pleuropneumoniae* [1, 5, 6, 22]. Therefore, PCR methods based on the toxin genes have been developed to facilitate classical biology and biochemical examinations [4]. However, little is known about the hemolytic and cytotoxic activities of ApxIV. It is well known that ApxI and ApxIII are encoded by classical RTX genes in a *CABD* manner. The *A* gene encodes the structural toxin, which is activated by the product of the *C* gene and secreted via its own secretion system encoded by the *B* and *D* genes. However, the *apxII* gene is truncated in all *A. pleuropneumoniae* serovars, having only *CA* genes and missing the *BD* genes. ApxII can be secreted via the secretion system of *apxI* genes but not via that of *apxIII* genes. Few studies have reported a *CABD* manner for *apxIV* genes. The *apx* gene profiles are inherent to a given serovar [1]. Differences in *apx* gene profiles are strongly related to differences in pathogenicity between serovars [6].

It has been shown that serotyping of *A. pleuropneumoniae* isolates is useful for understanding the epidemiology of an outbreak and for preparing vaccines for the control of the disease [3, 6]. Cross-reactions between some serovars (1, 9 and 11; 4 and 7; and 3, 6, 8 and 15) are usually observed in slide agglutination and agar gel precipitation (AGP) tests, which prevent accurate and rapid typing of field strains [3, 6, 18]. To overcome such problems, many PCR methods based on the toxin genes and capsule loci have been developed to enable precise serotyping [2, 4, 7, 25, 27].

Serovar 15 isolates generally harbor *apxIBD*, *apxIIICA* and *apxIICABD* genes. Very recently, in Australia, 17 of 40 (42.5%) serovar 15 isolates examined reportedly lacked, as expected, the *apxIIICA* genes [26]. It is not presently clear whether serovar 15 isolates lacking the *apxIIICA* genes exist in Japan, where serovar 2 is the most predominant, followed by serovars 1, 5 and 15 [8, 14, 16, 23].

In 2015, six fattening pigs at approximately 180 days of age suffering from acute pleuropneumonia suddenly died. The farm

*Correspondence to: To, H.: toho@nibs.or.jp

©2019 The Japanese Society of Veterinary Science



This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License. (CC-BY-NC-ND 4.0: <https://creativecommons.org/licenses/by-nc-nd/4.0/>)

was located in the Chubu region. Gross lesions of the pigs' lungs included extensive necrosis of the caudal lobes with many fibrinous and fibrous adhesions to the thoracic wall. The gross lesions resembled those caused by *A. pleuropneumoniae*. *A. pleuropneumoniae* serovar 15 was isolated from the lung lesions of all six dead pigs. Additionally, Porcine Circovirus 2 was identified, but other bacteria, Porcine Reproductive and Respiratory Syndrome virus and Hog Cholera virus were not identified. The six *A. pleuropneumoniae* isolates had a toxin gene profile different from that of the serovar 15 reference strain HS143.

In this study, we report the isolation and characterization of an atypical variant of *A. pleuropneumoniae* serovar 15 lacking the *apxIIICA* genes in Japan.

Six atypical strains of serovar 15 (A1, A2, A3, A4, A5, A6) isolated from the lung lesions of pneumonic naturally infected, dead pigs from farm A, and five representative field strains (B1, B2, C1, C2, D1) of serovar 15 isolated from farms B, C and D in Japan were used in this study. Isolates A1-6, B1-2 and C1-2 were thought to belong to the different clonal groups because the strains were isolated from the same farm at the same time and showed the same genotyping results. These farms are located in non-adjacent prefectures. Seventeen strains of *A. pleuropneumoniae* (serovar 1, 4047; serovar 2, CCM5870; serovar 3, S1421; serovar 4, M62; serovar 5a, K17; serovar 5b, L20; serovar 6, Femo; serovar 7, WF83; serovar 8, 405; serovar 9, CVJ13261; serovar 10, D13039; serovar 11, 56153; serovar 12, 1096; serovar 13, N273; serovar 14, 3096; serovar 15, HS143; and serovar 16, A-85/14) were used as reference strains for the AGP test.

The *A. pleuropneumoniae* isolates were cultured in chocolate II agar (BD, Becton, Dickinson Co., Detroit, MI, U.S.A.) or in heart infusion (HI) medium (BD) supplemented with 0.3% yeast extract (dried yeast extract-S, Nippon Seiyaku, Tokyo, Japan) and 0.005% β -nicotinamide adenine dinucleotide (NAD) (Oriental Yeast, Tokyo, Japan). *Escherichia coli* XL1-Blue were grown in Luria-Bertani medium (BD, Becton, Dickinson Co.). When appropriate, the medium was supplemented with ampicillin (50 μ g/ml) or isopropyl- β -D-thiogalactopyranoside (1 mM).

Phenotypic species identification of the field isolates (one isolate each from farms A, B, C and D) and a reference strain HS143 was achieved using the ID Test HN 20 Rapid Kit (Nissui Pharmaceutical Co., Tokyo, Japan). Biovars of *A. pleuropneumoniae* isolates were classified based on the requirement of NAD for growth [14]. A test for growth dependency of NAD was conducted to determine whether the isolate could grow on agar plates containing the above-mentioned medium without NAD. The CAMP and hemolysis assays were assessed on sheep blood agar plates supplemented with 0.005% NAD as described previously [11]. Serotyping of the Japanese isolates was carried out by the AGP test using rabbit antisera against serovars 1–16 of *A. pleuropneumoniae* as described previously [14]. The *Apx* toxin gene PCR profiling (detecting the presence of *apxICA*, *apxIBD*, *apxIICA*, *apxIIICA*, *apxIIIBD* and *apxIVA* genes) and capsule loci typing (*cps3*-, *6*-, *8*- and *cpsxD15*-specific PCR) were performed as previously described [4, 7, 14, 25, 27].

The 292-bp fragment of the *cpsxD* gene of serovar 15 that was amplified from the reference strain HS143 and 11 Japanese strains was cleaned up using ExoSAP-I (Amersham Pharmacia Biotech, Uppsala, Sweden) and sequenced on a 3730 \times 1 DNA Analyzer (Applied Biosystems, Foster City, CA, U.S.A.), using a BigDye Terminator cycle sequencing kit (Applied Biosystems). Additionally, an amplified DNA fragment encoding the full-length *apxIIIA* genes was ligated into the plasmid pGEM-T Easy (Promega, Madison, WI, U.S.A.), and transformed into *E. coli* XL1-Blue by electroporation. DNA sequences from recombinant plasmids were determined by a primer-walking procedure, starting with M13 vector primers, and were analyzed with the GENETYX-MAX program, version 12 (SDC, Tokyo, Japan), and the EditSeq and SeqMan programs of the DNASTar software package (DNASTAR Inc., Madison, WI, U.S.A.). Nucleotide and amino acid sequence alignments were performed with the CLUSTAL W program (<http://www.ddbj.nig.ac.jp>) as described previously [24].

Expression of *Apx* toxins (*ApxII* and *ApxIII*) of *A. pleuropneumoniae* was examined using an immunoblot procedure based on polyclonal anti-r*ApxIIA* and anti-r*ApxIIIA* antibodies as described previously [24]. Five-hour cultures of the reference (HS143) and five field (A1, A2, B1, C1 and D1) isolates were centrifuged and the resulting supernatant was precipitated with 10% (v/v) trichloroacetic acid (Wako, Osaka, Japan). The resulting pellets were resuspended in PBS and sample buffer, and were used to examine the expression of the toxin. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting were performed as described elsewhere [24].

The *Apx* toxin gene profile observed by the PCR typing system was confirmed by Southern blot analysis. Two of the six isolates (A1 and A2) from farm A, one isolate each (B1, C1 and D1) from farms B, C, and D, and a reference strain HS143 were included. The procedures were carried out as described previously [15, 17]. Briefly, genomic DNA was digested with the *HindIII* restriction enzyme and separated by size on a 1% agarose gel. After transferring to positively charged nylon membranes, hybridization with digoxigenin-labeled probes was performed according to the manufacturer's instructions in a rotating hybridization oven at 42°C. Probes were generated by PCR as used for *apx* gene profiling [4, 14] by incorporation of digoxigenin-11-2'-deoxyuridine 5'-triphosphate. Post-hybridization washing steps were performed twice for 30 min at room temperature in 2 \times saline-sodium citrate (SSC), 1 \times SSC and 0.1 \times SSC, respectively, by using DIG Wash and Block Buffer Set (Roche, Basel, Switzerland).

The procedures used to determine the pathogenicity of the 4 field isolates (A1, B1, C1 and D1) and a reference strain HS143 in mice were carried out as described previously [17]. Briefly, specific-pathogen-free female ddY mice aged 8 weeks (Japan SLC Co., Ltd., Shizuoka, Japan) were used. The field isolates (A1, B1, C1 and D1) and the reference strain HS143 were grown with shaking at 37°C for 4 hr in HI broth supplemented with 0.005% β -NAD. The culture was serially diluted ten-fold in HI broth and supplemented with 10% mucin (Sigma, St. Louis, MO, U.S.A.). Each of ten mice was injected intraperitoneally with the diluted cultures. Mouse mortality was monitored for the following 7 days. The animals used in this study were cared for in accordance with the guidelines for animal treatment of the Nippon Institute for Biological Science, which conform to the standard principles of laboratory animal care.

Table 1. Phenotypic and genotypic characteristics of atypical and typical *Actinobacillus pleuropneumoniae* strains

	A1	A2	A3	A4	A5	A6	B1	B2	C1	C2	D1	HS143
Farm	A						B		C		D	
Prefecture	A'						B'		C'		D'	
Region	Chubu						Kyusyu		Chubu		Kanto	
Year	2015						2013		2017		2007	
Phenotyping ^{a)}												
β-NAD	+	NT ^{b)}	NT	NT	NT	NT	+	NT	+	NT	+	+
Indole	-	NT	NT	NT	NT	NT	-	NT	-	NT	-	-
Oxidase	+	NT	NT	NT	NT	NT	+	NT	+	NT	+	+
Mannitol	+	NT	NT	NT	NT	NT	+	NT	+	NT	+	+
Urease	+	NT	NT	NT	NT	NT	+	NT	+	NT	+	+
Catalase	+	NT	NT	NT	NT	NT	+	NT	+	NT	+	+
Hemolysis	-	NT	NT	NT	NT	NT	+	NT	+	NT	+	+
Genotyping												
<i>apxICA</i>	-	-	-	-	-	-	-	-	-	-	-	-
<i>apxIBD</i>	+	+	+	+	+	+	+	+	+	+	+	+
<i>apxIICA</i>	-	-	-	-	-	-	-	-	+	+	+	+
<i>apxIIICA</i>	+	+	+	+	+	+	+	+	+	+	+	+
<i>apxIIIIBD</i>	+	+	+	+	+	+	+	+	+	+	+	+
<i>apxIVA</i>	+	+	+	+	+	+	+	+	+	+	+	+
<i>cps3</i>	-	-	-	-	-	-	-	-	-	-	-	-
<i>cps6</i>	-	-	-	-	-	-	-	-	-	-	-	-
<i>cps8</i>	-	-	-	-	-	-	-	-	-	-	-	-
<i>cps15</i>	+	+	+	+	+	+	+	+	+	+	+	+

a) For phenotypic tests, one strain each from farms A, B, C and D, and a reference strain HS143 were used. b) Not tested.

The phenotyping results of four representative field isolates (A1, B1, C1 and D1) and a reference strain HS143 are shown in Table 1. The seven-digit biochemical profile generated by the ID test HN-20 Rapid kit of these four strains was #7107351, and identified as *A. pleuropneumoniae*. Although three *A. pleuropneumoniae* isolates (B1, C1 and D1) and the reference strain HS143 were weakly hemolytic, isolate A1 was not hemolytic. The genotyping results of eleven field isolates (A1-A6, B1, B2, C1, C2 and D1) are shown in Table 1. These field strains were identified as serovar 15 by the AGP test and serovar 15-specific PCR test [25]. Apx toxin gene PCR profiling showed that five *A. pleuropneumoniae* isolates (B1, B2, C1, C2 and D1) contained the same *apx* gene combination as the serovars 2, 4, 6, 8 and 15 (the presence of *apxIIICA*, *apxIIICA*, *apxIBD* and *apxIIIIBD* genes), whereas six isolates from farm A contained only *apxIIICA*, *apxIBD*, and *apxIIIIBD*, but not *apxIICA* genes. Additionally, the analysis of toxin protein production showed that the typical isolates expressed ApxII and ApxIII, but the atypical isolates expressed only ApxIII. Nucleotide sequence similarity of the 292-bp fragment of the *cpxsD* gene of serovar 15 from eleven field isolates and reference strain HS143 was 100%.

Sequence analysis revealed that the size of the putative *apxIIIA* ORFs for representative field isolates of serovar 15 was 3,159 nucleotides, similar to those of serovars 6, 8 and 15, which encode proteins of 1,052 amino acids. The amino acid sequence similarities of ApxIIIA within serovar 15 were 99.71 to 100%.

Southern blot analysis confirmed the results of Apx toxin gene PCR profiling, in which six serovar 15 isolates from farm A harbored *apxIIICA*, *apxIBD* and *apxIIIIBD* genes, but not *apxIICA* genes, as shown in Fig. 1. Immunoblot analysis revealed that the field isolates lacking *apxIICA* genes did not produce the ApxII toxin (Fig. 2).

The results of the mouse pathogenicity tests of field isolates are shown in Table 2. The mouse LD₅₀ value of isolates B1, C1, D1 and the reference strain of serovar 15 were similar (10^{8.3}, 10^{8.2}, 10^{7.6} and 10^{7.9} CFU, respectively). The mouse LD₅₀ value of isolate A1 (10^{9.0} CFU) was higher than those of the other isolates. The results suggested that the pathogenicity of atypical isolate A1 (lacking *apxIICA* genes) seemed to be lower than that of the typical serovar 15 isolates in mice.

In Japan, disease due to *A. pleuropneumoniae* serovar 15 has been found in Chiba, Ehime, Fukuoka, Hokkaido and Kagawa prefectures of Japan, and this serovar is the fourth most predominant serovar behind serovars 2, 1 and 5 [8, 14, 16, 23]. Recently, there have been several reports on field strains of *A. pleuropneumoniae* that do not possess the typical toxin profile associated with their serovar [1, 9, 13, 15, 26]. An earlier report [13] that used immunoblot analysis with monoclonal antibodies to detect toxin production reported that one field strain each of serovars 2 and 9 expressed ApxII only, while two field strains of serovar 7 expressed both ApxII and ApxIII. Recently, Kuhnert *et al.* [15] reported that some field strains of serovar 3 isolated in Germany and Switzerland did not contain the *apxIICA* genes, and Ito *et al.* [9] found a Japanese strain of serovar 12 harboring *apxIICA*, *apxIIICA* and *apxIIIIBD* genes, but not *apxICA* or *apxIBD* genes. A more recent study [26] that used the *apx*-based PCR methods to examine the Apx toxin profiles reported that some Australian field isolates of serovar 15 did not contain *apxIICA* genes. Our results revealed the existence of atypical *A. pleuropneumoniae* serovar 15 isolates lacking the *apxIICA* genes on a Japanese farm. Our

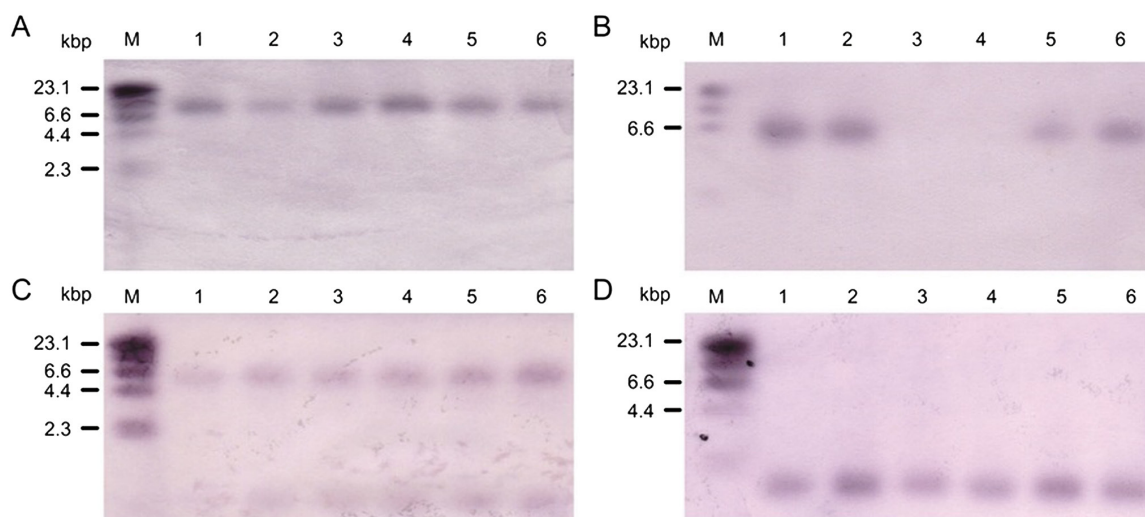


Fig. 1. Southern blot analysis of genomic DNA from *Actinobacillus pleuropneumoniae* serovar 15 isolates. Genomic DNA was digested with *Hind*III and separated by electrophoresis on an agarose gel. After transferring to nylon membranes, the DNA was hybridized with specific digoxigenin-labeled probes. **A**, hybridization with *apxIBD* probe. **B**, hybridization with *apxIIICA* probe. **C**, hybridization with *apxIIIICA* probe. **D**, hybridization with *apxIIIIBD* probe. M=digoxigenin-labeled marker in kb (Roche, Basel, Switzerland); lane 1: *A. pleuropneumoniae* serovar 15 isolate B1; lane 2: *A. pleuropneumoniae* isolate C1; lane 3: *A. pleuropneumoniae* isolate A1; lane 4: *A. pleuropneumoniae* isolate A2; lane 5: *A. pleuropneumoniae* isolate D1; lane 6: *A. pleuropneumoniae* strain HS143.

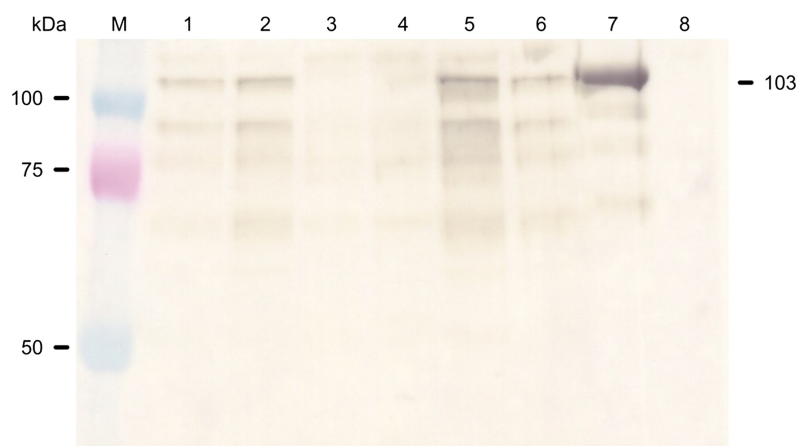


Fig. 2. Immunoblot analysis of the concentrated supernatants of *A. pleuropneumoniae* isolates (native Apx) and recombinant ApxII and ApxIII probed with anti-rApxII rabbit antisera. The concentrated supernatants of *A. pleuropneumoniae* isolates B1 (lane 1), C1 (lane 2), A1 (lane 3), A2 (lane 4), D1 (lane 5), HS143 (lane 6), rApxII (lane 7) and rApxIII (lane 8). M, molecular masses in kilodaltons (kDa). Number on the right is the molecular mass of rApxII in kilodaltons.

Table 2. Mouse pathogenicity of 5 *Actinobacillus pleuropneumoniae* strains

Strain ^{a)}	LD ₅₀ ^{b)}
A1	9.0
B1	8.3
C1	8.2
D1	7.6
HS143	7.9

a) For pathogenicity tests, one strain each from farms A, B, C and D, and a reference strain HS143 were used. b) Log CFU.

results demonstrated that: (i) six field isolates examined harbor *apxIBD*, *apxIIIICA* and *apxIIIIBD* genes but not *apxIIICA* genes; (ii) these atypical isolates express ApxIII but not ApxII; and (iii) the virulence of the atypical isolate toward mice seemed to be lower than that of the typical serovar 15 isolates.

Our observation that the pathogenicity of the atypical isolates seemed to be lower than that of the typical serovar 15 isolates is consistent with data from previous reports by Prideaux *et al.* [19] and Rycroft *et al.* [20]. Prideaux *et al.* [19] demonstrated that a serovar 7 strain with an inactivated *apxIIC* gene has reduced virulence as compared with the parent strain. Rycroft *et al.* [20] reported that a hemolysin-deficient serovar 2 mutant which still produces ApxIII, but not ApxII, was able to induce the same lung lesions in infected pigs as the wild type strain. The clinical signs in pigs infected with the mutant seemed to be less severe compared to the parent strain. The lack of *apxIIICA* genes may contribute to the variation in virulence of serovar 15 observed in the field [26].

The mechanism of the loss of the *apxIIICA* genes in the atypical strain was not defined in this study. Nevertheless, data from a report by Beck *et al.* [1] suggested that the toxin gene might have been deleted at hot spots of recombination or via the action of insertion sequence-like elements. Our results, together with data reported by Yee *et al.* [26] show the appearance of the atypical isolates of serovar 15 in Japan and Australia where the incidence of the disease due to serovar 15 seems to be increasing in recent years. Therefore, further research is necessary to find out whether the appearance of serovar 15 harboring only *apxIV* and *apxIII* genes reflects a new emerging variant of *A. pleuropneumoniae*.

In conclusion, we report the isolation and characterization of atypical serovar 15 isolates devoid of the *apxIIICA* genes in Japan. The atypical field isolate of serovar 15 seems to be less virulent in mice than the typical isolates, but it was isolated from lung lesions of dead fattening pigs. The contribution of ApxII to virulence remains to be further investigated although ApxII is one of the virulence factors of *A. pleuropneumoniae*. Monitoring the presence of serovar 15 isolates devoid of the *apxIIICA* genes may be helpful for resolving the question.

ACKNOWLEDGMENTS. The authors would like to thank Professor Laszlo Fodor and Dr. Rita Sárközi (University of Veterinary Medicine, Budapest, Hungary) for providing strain A-85/14, Dr. Conny Turni (University of Queensland, Australia) for a critical review of the manuscript, and Mr. Torata Ogawa (Fukuoka Prefecture Central Livestock Hygiene Service Center, Fukuoka, Japan) and the many veterinarians at pig farms of the prefectures for their invaluable help. We thank Ms. Miyuki Fujino and Ms. Michiha Kon (Nippon Institute for Biological Science) for their advice on the Southern blot assay.

REFERENCES

1. Beck, M., van den Bosch, J. F., Jongenelen, I. M., Loeffen, P. L., Nielsen, R., Nicolet, J. and Frey, J. 1994. RTX toxin genotypes and phenotypes in *Actinobacillus pleuropneumoniae* field strains. *J. Clin. Microbiol.* **32**: 2749–2754. [Medline]
2. Bossé, J. T., Li, Y., Sárközi, R., Fodor, L., Lacouture, S., Gottschalk, M., Casas Amoribiet, M., Angen, Ø., Nedbalcova, K., Holden, M. T. G., Maskell, D. J., Tucker, A. W., Wren, B. W., Rycroft, A. N., Langford P. R., BRADPIT consortium 2018. Proposal of serovars 17 and 18 of *Actinobacillus pleuropneumoniae* based on serological and genotypic analysis. *Vet. Microbiol.* **217**: 1–6. [Medline] [CrossRef]
3. Dubreuil, J. D., Jacques, M., Mittal, K. R. and Gottschalk, M. 2000. *Actinobacillus pleuropneumoniae* surface polysaccharides: their role in diagnosis and immunogenicity. *Anim. Health Res. Rev.* **1**: 73–93. [Medline] [CrossRef]
4. Frey, J., Beck, M., van den Bosch, J. F., Segers, R. P. and Nicolet, J. 1995. Development of an efficient PCR method for toxin typing of *Actinobacillus pleuropneumoniae* strains. *Mol. Cell. Probes* **9**: 277–282. [Medline] [CrossRef]
5. Frey, J., Bosse, J. T., Chang, Y. F., Cullen, J. M., Fenwick, B., Gerlach, G. F., Gygi, D., Haesebrouck, F., Inzana, T. J., Jansen, R., Kamp, E. M., Macdonald, J., MacInnes, J. I., Mittal, K. R., Nicolet, J., Rycroft, A. N., Segers, R. P., Smits, M. A., Stenbaek, E., Struck, D. K., van den Bosch, J. F., Willson, P. J. and Young, R. 1993. *Actinobacillus pleuropneumoniae* RTX-toxins: uniform designation of haemolysins, cytolysins, pleurotoxin and their genes. *J. Gen. Microbiol.* **139**: 1723–1728. [Medline] [CrossRef]
6. Gottschalk, M. 2012. Actinobacillosis. pp. 653–669. In: Diseases of Swine, 10th ed. (Zimmerman, J. J., Karriker, L., Ramirez, A., Schwartz, K. J. and Stevenson, G. W. eds), Wiley, Hoboken.
7. Gram, T., Ahrens, P., Andreassen, M. and Nielsen, J. P. 2000. An *Actinobacillus pleuropneumoniae* PCR typing system based on the *apx* and *omIA* genes—evaluation of isolates from lungs and tonsils of pigs. *Vet. Microbiol.* **75**: 43–57. [Medline] [CrossRef]
8. Ito, H. 2013. Recent topics of serotypes/serotyping and host specificity of *Actinobacillus pleuropneumoniae*. *Proc. Jpn. Pig Vet. Soc* **61**: 14–21 (in Japanese).
9. Ito, H. and Matsumoto, A. 2015. Isolation and genetic characterization of an *Actinobacillus pleuropneumoniae* serovar K12:O3 strain. *J. Vet. Diagn. Invest.* **27**: 102–106. [Medline] [CrossRef]
10. Ito, H. and Sueyoshi, M. 2015. The genetic organization of the capsular polysaccharide biosynthesis region of *Actinobacillus pleuropneumoniae* serotype 15. *J. Vet. Med. Sci.* **77**: 483–486. [Medline] [CrossRef]
11. Jansen, R., Briaire, J., Kamp, E. M., Gielkens, A. L. J. and Smits, M. A. 1995. The CAMP effect of *Actinobacillus pleuropneumoniae* is caused by Apx toxins. *FEMS Microbiol. Lett.* **126**: 139–143. [Medline] [CrossRef]
12. Kamp, E. M., Stockhofe-Zurwieden, N., van Leengoed, L. A. and Smits, M. A. 1997. Endobronchial inoculation with Apx toxins of *Actinobacillus pleuropneumoniae* leads to pleuropneumonia in pigs. *Infect. Immun.* **65**: 4350–4354. [Medline]
13. Kamp, E. M., Vermeulen, T. M., Smits, M. A. and Haagsma, J. 1994. Production of Apx toxins by field strains of *Actinobacillus pleuropneumoniae* and *Actinobacillus suis*. *Infect. Immun.* **62**: 4063–4065. [Medline]
14. Koyama, T., To, H. and Nagai, S. 2007. Isolation of *Actinobacillus pleuropneumoniae* serovar 15-like strain from a field case of porcine pleuropneumonia in Japan. *J. Vet. Med. Sci.* **69**: 961–964. [Medline] [CrossRef]
15. Kuhnert, P., Rohde, J. and Korczak, B. M. 2011. A new variant of *Actinobacillus pleuropneumoniae* serotype 3 lacking the entire *apxII* operon. *J. Vet. Diagn. Invest.* **23**: 556–559. [Medline] [CrossRef]
16. Morioka, A., Asai, T., Nitta, H., Yamamoto, K., Ogikubo, Y., Takahashi, T. and Suzuki, S. 2008. Recent trends in antimicrobial susceptibility and the presence of the tetracycline resistance gene in *Actinobacillus pleuropneumoniae* isolates in Japan. *J. Vet. Med. Sci.* **70**: 1261–1264. [Medline] [CrossRef]
17. Nagai, S., Takahashi, K., Mitsui, T. and Yagihashi, T. 1993. *hlyIA*-like sequence present in a genetic locus with low homology to *appA* in *Actinobacillus pleuropneumoniae* serotype 10. *J. Vet. Med. Sci.* **55**: 813–819. [Medline] [CrossRef]
18. Perry, M. B., Altman, E., Brisson, J. R., Beynon, L. M. and Richards, J. C. 1990. Structural characteristics of the antigenic capsular polysaccharides and lipopolysaccharides involved in the serological classification of *Actinobacillus (Haemophilus) pleuropneumoniae* strains. *Serodiagn. Immunother. Infect. Dis.* **4**: 299–308. [CrossRef]
19. Prideaux, C. T., Lenghaus, C., Krywult, J. and Hodgson, A. L. 1999. Vaccination and protection of pigs against pleuropneumonia with a vaccine strain of *Actinobacillus pleuropneumoniae* produced by site-specific mutagenesis of the ApxII operon. *Infect. Immun.* **67**: 1962–1966. [Medline]
20. Rycroft, A. N., Williams, D., McCandlish, I. A. and Taylor, D. J. 1991. Experimental reproduction of acute lesions of porcine pleuropneumonia with a haemolysin-deficient mutant of *Actinobacillus pleuropneumoniae*. *Vet. Rec.* **129**: 441–443. [Medline] [CrossRef]

21. Sárközi, R., Makrai, L. and Fodor, L. 2015. Identification of a proposed new serovar of *Actinobacillus Pleuropneumoniae*: Serovar 16. *Acta Vet. Hung.* **63**: 444–450. [[Medline](#)] [[CrossRef](#)]
22. Schaller, A., Kuhn, R., Kuhnert, P., Nicolet, J., Anderson, T. J., MacInnes, J. I., Segers, R. P. and Frey, J. 1999. Characterization of *apxIVA*, a new RTX determinant of *Actinobacillus pleuropneumoniae*. *Microbiology* **145**: 2105–2116. [[Medline](#)] [[CrossRef](#)]
23. Shimazaki, Y., Uchiyama, M., Kojima, A., Hirano, F., Sasaki, Y. and Nagai, H. 2016. Cross immunity in mice vaccinated by porcine pleuropneumonia challenge exposure with strains of *Actinobacillus pleuropneumoniae* serotype 15 isolated in Japan. *Ann. Rep. Natl. Vet. Assay Lab.* **53**: 217–227 (in Japanese with English summary).
24. To, H., Nagai, S., Iwata, A., Koyama, T., Oshima, A. and Tsutsumi, N. 2016. Genetic and antigenic characteristics of ApxIIA and ApxIIIA from *Actinobacillus pleuropneumoniae* serovars 2, 3, 4, 6, 8 and 15. *Microbiol. Immunol.* **60**: 447–458. [[Medline](#)] [[CrossRef](#)]
25. Turni, C., Singh, R., Schembri, M. A. and Blackall, P. J. 2014. Evaluation of a multiplex PCR to identify and serotype *Actinobacillus pleuropneumoniae* serovars 1, 5, 7, 12 and 15. *Lett. Appl. Microbiol.* **59**: 362–369. [[Medline](#)] [[CrossRef](#)]
26. Yee, S., Blackall, P. J. and Turni, C. 2018. Genetic diversity and toxin gene distribution among serovars of *Actinobacillus pleuropneumoniae* from Australian pigs. *Aust. Vet. J.* **96**: 17–23. [[Medline](#)] [[CrossRef](#)]
27. Zhou, L., Jones, S. C., Angen, Ø., Bossé, J. T., Nash, J. H., Frey, J., Zhou, R., Chen, H. C., Kroll, J. S., Rycroft, A. N. and Langford, P. R. 2008. Multiplex PCR that can distinguish between immunologically cross-reactive serovar 3, 6, and 8 *Actinobacillus pleuropneumoniae* strains. *J. Clin. Microbiol.* **46**: 800–803. [[Medline](#)] [[CrossRef](#)]