**NOTE**  Bacteriology

**Isolation of *Actinobacillus pleuropneumoniae* Serovar 15-Like Strain from a Field Case of Porcine Pleuropneumonia in Japan**

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**ABSTRACT.** An *Actinobacillus pleuropneumoniae* strain isolated from a field case of porcine pleuropneumonia in Japan, was closely related to a reference strain of serovar 15, which is a newly proposed serovar according to an analysis of field isolates originating from Australia. The isolate had biological and biochemical properties consistent with *A. pleuropneumoniae* biovar 1, and reacted strongly to a rabbit antiserum raised against a reference strain of serovar 15 in an agar gel precipitation test. The nucleotide sequence of a hyper variable region in the 16S RNA gene of the isolate was identical to that of the reference strain of serovar 15. The isolate possessed *A. pleuropneumoniae*-RTX toxin (Apx) II, III, and IV genes, consistent with serovar 15. Its virulence in mice was lower than that of ApxI-bearing strains but higher than that of other ApxIII-bearing strains. This is the first report describing the isolation of *A. pleuropneumoniae* serovar 15-like strain from a country or region other than Australia.

**KEY WORDS:** *Actinobacillus pleuropneumoniae*, serotyping, serovar 15.

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*Actinobacillus pleuropneumoniae* is the causative agent of porcine pleuropneumonia, an economically important disease of pigs in many countries [15]. A knowledge of which serovars of *A. pleuropneumoniae* are prevalent in a region or on a farm is important in understanding the epidemiological status of the disease and in selecting antibiotics to treat the disease [1]. *A. pleuropneumoniae* has been serotyped into 14 serovars: serovars 1 to 12, mainly of biovar 1 (growth dependent on β-nicotinamide-adenine dinucleotide (NAD)), and serovars 13 and 14 of biovar 2 (growth independent of NAD). Recently, a new serovar, 15, was proposed by Blackall et al. [4] based on an analysis of nine Australian isolates which had been erroneously assigned to serovar 12. However, there was no report describing the isolation of serovar 15-like strain in a country or part of the world other than Australia.

In Japan, serovars 2, 1, and 5 are the most prevalent forms of *A. pleuropneumoniae*, in decreasing order, accounting for 95% of field isolates [6]. However, other serovars have also been isolated from sporadic outbreaks of pleuropneumonia. In this report, we describe the isolation and partial characterization of *A. pleuropneumoniae* which is closely related to a serovar 15 reference strain, from a field case of porcine pleuropneumonia in Japan.

The *A. pleuropneumoniae* isolate was obtained from the lungs of a fattening pig approximately 150 days of age suffering from acute pleuropneumonia. The herd, where the pig was raised, was located in the Kanto area and consisted of approximately 600 sows with a farrow-to-finish production system. In October 2003, approximately 15% of fattening pigs of the herd died of acute pleuropneumonia. The mortality reached a peak at around 120–150 days of age.

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Reference strains of *A. pleuropneumoniae*, a serovar 1 strain 4074, a serovar 3 strain S1421, a serovar 7 strain WF83, and a serovar 10 strain 13039, were described previously [11]. A reference strain of serovar 15, HS143, was provided by Dr. P. J. Blackall (Animal Research Institute, Yeerongpilly, Australia). *A. pleuropneumoniae* was grown in heart infusion broth (Infusion broth, BBL, Cockesville, MD, U.S.A.) supplemented with 0.3% yeast extract (Dried yeast extract-S, Nippon Seiyaku Co., Ltd., Tokyo, Japan) and 0.0025% β-nicotinamide-adenine dinucleotide (NAD; Oriental Yeast Co., Ltd., Tokyo, Japan).

The isolate, designated NBAP008, was identified as belonging to *A. pleuropneumoniae* biovar 1 by conventional phenotypic tests. A test for growth dependency of NAD was conducted to determine if the isolate can grow on agar plates containing the above mentioned medium without NAD.

An agar gel precipitation test (AGP) was performed as described by Molner [9]. Antisera to serovars 1 to 12 and 15 of *A. pleuropneumoniae* biovar 1 were produced in rabbits according to the protocol described by Mittal et al. [8].

A DNA fragment encoding the 16S rRNA gene from position 8 to 926 (919 bp) was amplified by PCR as described elsewhere [2]. The nucleotide sequence was determined by our original PCR. A homology-based search of the GenBank/EMBL/DDBJ database was done using the BLAST programs (http://www.ddbj.nig.ac.jp).

The presence of the three Apx structural genes, *apxIA*, *apxIIA*, and *apxIII*, was determined by our original PCR. Total DNA was prepared from cultured bacteria using Instagene Matrix (Biorad, Hercules, CA, U.S.A.). The fol-
Following oligonucleotide primer pairs were selected and commercially synthesized for use in the PCR: 5’-CTTAGAAGATACCTTTGAAT-3’ and 5’-GACGTTACTCCTTACTGTAG-3’ for a 1,220-bp apxIA fragment; 5’-CTACCGTTATTGATGGCGGGACGGACATG-3’ and 5’-TCCTAAATTATTCCTAAAGTCTGAGGAAGA-3’ for a 932-bp apxIIA fragment; and 5’-TCTACAGAAGC-GAAAATCAT-3’ and 5’-CCAATGGACTGAT-TGCTAAA-3’ for a 1,120-bp apxIIIA fragment. The PCR was carried out as described previously [10] except that the denaturation temperature was 88°C. For amplification of the toxin secretion genes, apxIBD, apxIIBD, and apxIIIBD, and the other toxin structural gene apxIVA, primers and PCR conditions were applied as described elsewhere [5, 13].

The median lethal dose (LD50) in mice was determined as described previously [11]. The isolate NBAP008 was a small Gram-negative, coccobacillus. It was NAD-dependent, cytochrome oxidase-positive, catalase-positive, indole-negative, urease-positive, and phosphatase-positive, and could reduce nitrate. It was shown to ferment glucose, mannose, mannitol, sucrose and D-xylose, but not to ferment L-arabinose and trehalose. Based on these properties, NBAP008 was identified as A. pleuropneumoniae biovar 1. It did not show a clear zone of erythrocyte lysis on a sheep blood agar plate.

In the AGP test, antisera raised against serovars 1 to 12 of A. pleuropneumoniae biovar 1 did not react with NBAP008 with exception that a faint line of precipitation was obtained with an antiserum against serovar 7 (Fig. 1). In contrast, the antiserum raised against the reference strain of serovar 15 reacted strongly with the isolate.

To determine the phylogenetic relationship between NBAP008 and HS143 and other A. pleuropneumoniae serovar strains as well, the nucleotide sequence of a hyper variable region of the 16S rRNA gene of the isolate was determined and compared with those of HS143 and other A. pleuropneumoniae strains which are available in the sequence databases. A similarity analysis revealed that the nucleotide sequence of the isolate is identical to that of the reference strain of serovar 15, and is 98 to 99% identical to sequences of other serovar strains. A phylogenetic tree showed that NBAP008 is most closely related to the reference strain of serovar 15 among the serovars of A. pleuropneumoniae (Fig. 2). Next, we detected structural genes for Apx toxins (apxIA, apxIIA, apxIIIA and apxIVA) as well as genes associated with the secretion of Apx toxins (apxIBD, apxIIBD and apxIIIBD) by PCR, since the toxin-gene profile has been shown to be inherent to each serovars and the 12 serovars of A. pleuropneumoniae biovar 1 can be grouped into five different Apx toxin-gene patterns. [5]. As representatives of each toxin-gene group, the reference strains of serovars 1, 3, 7, 10 and 15 were used as controls.

The isolate NBAP008 was shown to possess Apx structural genes, apxIIA, apxIIIA, and apxIVA, but not apxIA, and Apx secretion genes, apxIBD and apxIIBD (Table 1). Thus, the toxin-gene profile was identical to that of one toxin-gene group including serovars 2, 4, 6, 8 and 15.

The LD50 of NBAP008 and HS143 in mice were calculated as 107.4 and 107.6 CFU, respectively.

Serological typing of A. pleuropneumoniae isolates is a difficult and demanding task. It was described that strong cross-reactions are sometimes observed between serovars 1, 9, and 11; 6 and 8; and 4 and 7 [12]. The serovar 15 strains obtained from Australian pigs were originally assigned to serovar 12 because of their cross-reactivity [3]. In the present study, we did not observe any cross reaction with the heat-treated antigen of NBAP008 and rabbit antiserum against serovars 1 to 12 except serovar 7 in the AGP test. To achieve a high degree of specificity for the relevant serovar, the immunization protocol in rabbits is important. In accordance with the originally described procedure, we injected five rabbits with formalin-killed whole cells of A. pleuropneumoniae via the intravenous route without any adjuvant. Antibody titers of the antisera were determined with homologous antigen and we simultaneously confirmed their specificity using heterologous serovar antigens. Normally, one or two of the five sera were available for the AGP serotyping test.

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Fig. 1. Results of an agar gel precipitation test. The heat extracted antigen of NBAP008 was placed in the center wells and rabbit antisera against 1 to 12 and 15 serovars of A. pleuropneumoniae biovar 1 reference strains were placed in the surrounding wells.
A. PLEUROPNEUMONIAE SEROVAR 15 LIKE STRAIN

The antiserum against serovar 7 weakly reacted with NBAP008. A cross reaction between serovars 7 and 15 was described previously [4]. However, it was clear that NBAP008 could not be assigned to serovar 7, because the toxin gene profile of serovar 7 is apxIIA, apxIVA, and apxIBD, but that of NBAP008 was apxIIA, apxIIIA, apxIVA, apxIBD, and apxIIIBD. It is well known that the RTX toxin genotypes are inherent to their serotype [5] and the isolate was shown to be classified into a toxin-gene-group of serovars 2, 4, 6, 8 and 15. Since antisera against serovars 2, 4, 6, and 8 did not cross-react with the isolate, the isolate could be assigned to serovar 15. Moreover, a reactivity of antisera against the serovar 15 reference strain HS143 to the homologous antigen was abolished by absorption with inactivated whole cells of the isolate NBAP008 (data not shown). The identical 16S RNA gene sequence of NBAP008 with that of serovar 15, and a phylogenetic analysis of these sequences from various A. pleuropneumoniae strains, also support the results of serotyping.

The mouse LD₅₀ value of the isolate NBAP008 and the reference strain of serovar 15 are very similar (10⁷.4 and 10⁷.6 CFU, respectively; Fig. 3). These values are lower than that of any other ApxIII-producing serovar (10⁸.3, 10⁸.9, 10⁷.9, 10⁹.6, and 10⁸.7 CFU for serovars 2, 3, 4, 6, and 8, respectively [11]) but higher than that of ApxI-producing serovars (10⁵.9, 10⁵.2, 10⁵.8, 10⁵.3, 10⁵.0, and 10⁵.9 CFU for serovars 1, 5a, 5b, 9, 10, and 11, respectively [11]). In our previous study, we found that the LD₅₀ value for mice is related to the type of toxin produced by the relevant strain [11]. The ApxI-bearing serovars appeared to be more virulent in mice than non-ApxI-bearing serovars. However, it is noteworthy that NBAP008 and HS143 were more virulent
than other ApxIII-bearing serovars in mice, suggesting that serovar 15 strains may also be highly virulent in pigs since some degree of relationship between virulence in mice and virulence in pigs has been indicated [7].

In Japan, the most prevalent serovars are 2, 1, and 5 (in decreasing order), but other serovars are also isolated occasionally. However, to the best of our knowledge, this is the first report describing the isolation of serovar 15-like strain from a pig reared in Japan, or in any other part of the world except Australia. The recognition of this new serovar has important implications for the prevention and control of porcine pleuropneumonia in Japan. Since bacterin-type vac
cines are effective only ag
ainst homologous serovars contained in the vaccine, the current vaccine consisting of serovars 1, 2, and 5 bacterins may not be effective against serovar 15. On the other hand, toxin-type vaccines (three kinds of vaccines are commercially available in Japan), are effective against a wide variety of A. pleuropneumoniae serovars [15], because Apx toxins are common among various serovar strains. The efficacy of one toxin-type vaccine was evaluated using an Australian serovar 15 strain [16]. More studies are needed to be clarified whether these toxin-type vaccines would be effective against this serovar 15-like strain isolated from a Japanese field as well.

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