Acta Veterinaria Hungarica 63 (4), pp. 444–450 (2015)
DOI: 10.1556/004.2015.041

IDENTIFICATION OF A PROPOSED NEW SEROVAR OF *ACTINOBACILLUS PLEUROPNEUMONIAE:* SEROVAR 16

Rita SÁRKÖZI*, László MAKRAI and László FODOR

Department of Microbiology and Infectious Diseases, Faculty of Veterinary Science, Szent István University, P.O. Box 22, H-1581 Budapest, Hungary

(Received 1 September 2015; accepted 28 October 2015)

Five *Actinobacillus pleuropneumoniae* strains isolated from pathological lesions of porcine pleuropneumonia in Hungary could not be assigned to any of the accepted 15 serovars. Using hyperimmune serum raised against these untypable-serovar *A. pleuropneumoniae* strains in rabbits, indirect haemagglutination tests proved that they form a distinct group and there is no cross-reaction between them and the type strains of *A. pleuropneumoniae*. All five strains harboured the toxin-associated genes for the production *(apxIA)* and secretion *(apxIB)* of ApxI, the gene for the expression of ApxII and the largest-size (2800 bp) *apxIV* gene. The carbon source utilisation pattern and the sequence analysis of the 16S rRNA gene confirmed the species identification of the suggested type strain, *A. pleuropneumoniae* A-85/14. A new serovar of *A. pleuropneumoniae* – serovar 16 – is proposed with *A. pleuropneumoniae* A-85/14 as reference strain.

Key words: *Actinobacillus pleuropneumoniae*, untypable strain, new serovar, serovar 16

Actinobacillus pleuropneumoniae is one of the most important agents of the swine respiratory disease complex; it causes haemorrhagic-necrotic pneumonia and fibrinous pleuritis in growing and fattening pigs throughout the world. It is a facultative pathogenic bacterium present on the mucous membranes of the porcine respiratory tract. In the presence of predisposing factors such as overcrowding, nutritional deficiencies, management problems or bacterial and viral infections, *A. pleuropneumoniae* can cause severe respiratory disease and losses. The bacterium has two biotypes: biotype 1 strains need nicotinamide adenine dinucleotide (NAD, V factor) for growth, while biotype 2 strains are not NAD dependent. Several virulence factors, among others Apx toxins, fimbriae, outer membrane proteins, ability of biofilm formation, presence of transporter systems and different enzymes are involved in the pathogenesis of pneumonia caused by *A. pleuropneumoniae* (Chiers et al., 2010; Grasteau et al., 2011).

*Corresponding author; E-mail: sarkozi.rita@aotk.szie.hu; Phone: 0036 (1) 251-9900; Fax: 0036 (1) 251-9260

On the basis of surface soluble capsular polysaccharide (CPS) and lipopolysaccharide (LPS) antigens, 15 serovars have been described (Nielsen, 1986*a,b*; Fodor et al., 1989; Nielsen et al., 1997; Blackall et al., 2002). Several methods have been used for serotyping *A. pleuropneumoniae* strains; these include slide agglutination, latex agglutination, complement fixation, indirect haemagglutination (IHA), coagglutination, ring precipitation and agar gel precipitation tests (Mittal et al., 1983*a*; Mittal et al., 1987; Molnár, 1990; Nielsen et al., 1997). Many authors have found that among the classical serotyping methods the IHA test is the most specific and sensitive (Mittal et al., 1983*b*; Nicolet et al., 1981; Nielsen and O'Connor, 1984; Nicolet, 1988). Cross-reactions make serotyping rather difficult, and such cross-reactions have been reported between serovars 1, 9 and 11, serovars 3, 6 and 8, and serovars 4 and 7 (Mittal et al., 1988; Zhou et al., 2008). Correlation between serovars and the virulence of *A. pleuropneumoniae* strains was described several years ago (Marsteller and Fenwick, 1999), which can be explained by the correlation between serovars and the pattern of toxin production (Kamp et al., 1994; Frey et al., 1995). On the basis of this correlation, classical serotyping methods are nowadays frequently replaced by the detection of toxin genes; however, the latter can be misguiding in the case of strains with atypical toxin production (Rayamajhi et al., 2005; Gottschalk, 2015). More specific molecular serotyping methods detect genes involved in the biosynthesis of capsular polysaccharides (Angen et al., 2008; Bossé et al., 2014; Marois-Crehan et al., 2014; Turni et al., 2014). Using amplified fragment length polymorphism (AFLP) analysis 20 clusters could be identified, and all clusters contained strains of a single serotype (Kokotovic and Angen, 2007).

Between 2012 and 2014, a total of 50 *A. pleuropneumoniae* strains isolated from clinical cases from different swine farms in Hungary were serotyped by the IHA test (data not presented), but five strains could not be assigned to any of the 15 known serovars of *A. pleuropneumoniae*. The aim of the present study was to examine these untypable strains and to present evidence about the existence of a new serovar of *A. pleuropneumoniae,* serovar 16.

Materials and methods

Bacteria

Five *A. pleuropneumoniae* field isolates of biotype 1 were included in the present study. Two of them were isolated from the lungs of slaughtered pigs showing typical lesions of pleuropneumonia. Three strains were isolated from postmortem cases of acute porcine pleuropneumonia, submitted to our laboratory from different swine farms. The strains originated from five different farms located in different parts of Hungary, with epidemiological connection existing only between two of them (some animals were transported from one farm to the

other). The reference strains of all 15 serovars of *A. pleuropneumoniae* were kindly provided by Dr. O. Angen (Danish Veterinary Laboratory, Copenhagen).

The *A. pleuropneumoniae* strains were isolated on Tryptone Soya Agar (TSA, Biolab Ltd. Budapest, Hungary) cross-inoculated with *Staphylococcus aureus,* and cultured on chocolate agar with added 50 μg/ml NAD (Biolab Ltd., Budapest, Hungary), both containing 10% defibrinated sheep blood. Cultures were incubated at 37 °C for 24 h in aerobic environment with the addition of 5% carbon dioxide. They were identified using standard methods (Barrow and Feltham, 1993). After identification, the isolated *A. pleuropneumoniae* strains were stored at –80 °C until further examination.

Production of antisera

Antisera were produced in rabbits against *A. pleuropneumoniae* reference strains of serovars 1–15 and against our five field strains as described by Biberstein (1978). Briefly, rabbits weighing about 3 kg were inoculated intravenously with one of the *A. pleuropneumoniae* strains. Bacteria from three chocolate agar cultures, incubated at 37 °C for 24 h, were suspended in 40 ml saline (approximately 3×10^{10} colony-forming units/ml) containing 0.5% formaldehyde. The suspension was allowed to stand at room temperature for 1 h, and then the bacterial cells were pelleted by centrifugation, resuspended in saline, and stored at 4 °C until used. The rabbits were inoculated intravenously with 0.5, 1, 2, 3, 3 and 3 ml of the suspension, respectively, 3–4 days apart. One week after the last injection, they were bled in general anaesthesia induced with ketamine and xylazine (Animal Experiment Licence No. 22.1/2703/003/2009). Sera were harvested and stored in small aliquots in a deep freezer at –20 °C until used.

Serological characterisation

The IHA test was performed as described by Biberstein (1978). All antisera produced against the 15 type strains of *A. pleuropneumoniae* and the five untypable *A. pleuropneumoniae* strains were examined with all type strains and the 5 untypable *A. pleuropneumoniae* strains. Reactions below a titre of 1:160 were neglected.

Detection of toxin genes by polymerase chain reaction (PCR)

The presence of *apxIA*, *apxIB*, *apxII*, *apxIII* and *apxIV* genes was examined using PCR. The primers and conditions for these PCR assays had been described by Rayamajhi et al. (2005).

Characterisation of A. pleuropneumoniae *strain A-85/14*

After the serological examinations, the biochemical characteristics and the nucleotide sequence of the 16S rRNA gene of a representative strain, *A. pleuropneumoniae* A-85/14 were determined. The utilisation of 95 carbon sources was examined using the Biolog system (BIOLOG Inc., California). The 16S rRNA gene was amplified by PCR using the universal primers, its nucleotide sequence was determined as described (Relman, 1993), and the *rrs* gene sequence was submitted to the GenBank database (Accession No. SUB1098860 A-85/14 KT763387).

Results

Using conventional methods, all five isolates proved to be *A. pleuropneumoniae* biotype 1 strains. They did not show any reactions with antisera raised against the 15 recognised type strains of *A. pleuropneumoniae* in the IHA test but they reacted with their homologous sera in titres of 1:2560 and 1:5120. All the five strains gave heterologous reactions in the same titre with the hyperimmune sera raised against the other four untypable *A. pleuropneumoniae* strains. None of the 15 type strains of *A. pleuropneumoniae* gave a reaction with the sera raised against the untypable *A. pleuropneumoniae* strain A-85/14.

All five strains harboured the genes for the production *(apxIA)* and secretion *(apxIB)* of ApxI, the gene for the expression of ApxII and the largest-size (2800 bp) *apxIV* gene.

Based upon the utilisation of 95 carbon sources, *A. pleuropneumoniae* strain A-85/14 was identified as *A. pleuropneumoniae*, as its carbon utilisation pattern showed 99% similarity with the database. Sequence analysis of the 16S rRNA detected 99% similarity with *A. pleuropneumoniae* (GenBank Accession No. SUB1098860 A-85/14 KT763387).

The *A. pleuropneumoniae* strain A-85/14 has been deposited in the Hungarian National Collection of Medical Bacteria (HNCMB) under the registration number of 96705.

Discussion

The five Hungarian isolates included in the present study were examined by the IHA test using hyperimmune sera raised against the 15 accepted serovars of *A. pleuropneumoniae*. As these strains did not show any reaction with the type sera, they proved to be untypable. Hyperimmune sera were produced in rabbits against the five untypable strains, and all these antisera showed both homologous and heterologous reactions of high titre within this group. None of the 15 type strains reacted with the sera produced against the untypable strains. Consequently, the five, formerly untypable *A. pleuropneumoniae* strains represent a

common and distinct serovar of *A. pleuropneumoniae*. Untypable *A. pleuropneumoniae* strains can be isolated from clinical samples; some of them could be allocated in clusters together with serotypable ones indicating loss of type-specific capsule polysaccharides (Kokotovic and Angen, 2007). In the case of our strains the capsule material was present and antibodies could be raised against them, so the cause of their being untypable was not the loss of antigens. Kokotovic and Angen (2007) described two clusters containing only untypable-serovar *A. pleuropneumoniae* strains, and they supposed that these untypable strains could represent additional serovars. Further investigations can reveal whether our strains belong to one of these clusters.

Four toxin genes, *apxIA*, *apxIB*, *apxII* and *apxIV* (2800 bp), were detected in all five strains; they can express ApxI and ApxII but not ApxIII, while ApxIV can be produced *in vivo* only (Schaller et al., 1999). This toxin gene pattern is typical of *A. pleuropneumoniae* serovars 5a and 5b (Rayamajhi et al., 2005). Using the IHA test, *A. pleuropneumoniae* serovars 5a and 5b did not react with antisera produced against the five untypable strains, so they do not belong to serovar 5a or 5b. The discrepancy between the results of the IHA test and serotyping based on the detection of toxin genes can be explained by the fact that the latter method does not detect genes responsible for antigen production; rather, it is based on the correlation between certain serovars and toxin production of the *A. pleuropneumoniae* strains. Although new molecular methods are widely used for serotyping *A. pleuropneumoniae* (Rayamajhi et al., 2005; Bossé et al., 2014; Marois-Crehan et al., 2014; Turni et al., 2014; Gottschalk, 2015), they cannot always replace traditional serotyping tests.

The results of utilisation of 95 carbon sources and the sequence analysis of the 16S rRNA gene confirmed that the representative *A. pleuropneumoniae* A-85/14 strain is a typical *A. pleuropneumoniae*.

Among the five *A. pleuropneumoniae* biotype 1 strains which proved to be untypable using hyperimmune sera raised against the 15 type strains of *A. pleuropneumoniae*, a new serovar was identified, represented by strain A-85/14, and all the five untypable *A. pleuropneumoniae* strains of our collection belong to this serovar. This serovar can be clearly differentiated from the other 15 serovars of *A. pleuropneumoniae* by IHA.

In conclusion, this newly characterised serovar represents a distinct serovar, and we recommend that it be designated as serovar 16 of *A. pleuropneumoniae* with *A. pleuropneumoniae* A-85/14 strain as the type strain. The recommended type strain has been deposited in the HNCMB under the accession number of 96705, and its *rrs* gene sequence has been submitted to GenBank (Accession No. SUB1098860 A-85/14 KT763387).

Acknowledgement

This work was supported by the Hungarian Scientific Research Fund (OTKA 84220 and OTKA 112826).

References

Angen, Ø., Ahrens, P. and Jessing, S. G. (2008): Development of multiplex PCR test for identification of *Actinobacillus pleuropneumoniae* serovars 1, 7 and 12. Vet. Microbiol. **132,** 312–318.

Barrow, G. I. and Feltham, R. K. A. (eds) (1993): Cowan and Steel's Manual for the Identification of Medical Bacteria. Cambridge University Press, Cambridge. 331 pp.

Biberstein, E. L. (1978): Biotyping and serotyping of *Pasteurella haemolytica*. In: Bergan, T. and Norris, J. R. (eds) Methods in Microbiology 10. Academic Press, London. pp. 253–269.

Blackall, P. J., Klaasen, H. L. B. M., Bosch, H. V. D., Kuhnert, P. and Frey, J. (2002): Proposal of a new serovar of *Actinobacillus pleuropneumoniae*: serovar 15. Vet. Microbiol. **84,** 47–52.

Bossé, J. T., Li, Y., Angen, O., Weinert, L. A., Chaudhuri, R. R., Holden, M. T., Williamson, S. M., Maskell, D. J., Tucker, A. W., Wren, B. W., Rycroft, A. N. and Langford, P. R. (2014): Multiplex PCR assay for unequivocal differentiation of *Actinobacillus pleuropneumoniae* serovars 1 to 3, 5 to 8, 10 and 12. J. Clin. Microbiol. **52,** 2380–2385.

Chiers, K., de Waele, T., Pasmans, F., Ducatelle, R. and Haesebrouck, F. (2010): Virulence factors of *Actinobacillus pleuropneumoniae* involved in colonisation, persistence and induction of lesions in its porcine host. Vet. Res. **41,** 65.

Fodor, L., Varga, J., Molnár, É. and Hajtós, I. (1989): Biochemical and serological properties of *Actinobacillus pleuropneumoniae* biotype 2 strains isolated from swine. Vet. Microbiol. **20,** 173–180.

Frey, J., Beck, M., Bosch, J. V. D., Segers, R. P. A. M. and Nicolet, J. (1995): Development of an efficient PCR method for toxin typing of *Actinobacillus pleuropneumoniae* strains. Mol. Cell Probes **9,** 277–282.

Gottschalk, M. (2015): The challenge of detecting herds sub-clinically infected with *Actinobacillus pleuropneumoniae*. Vet. J. **206,** 30–38. doi:10.1016/j.tvjl.2015.06.016

Grasteau, A., Tremblay, Y. D. N., Labrie, J. and Jacques, M. (2011): Novel genes associated with biofilm formation of *Actinobacillus pleuropneumoniae*. Vet. Microbiol. **153,** 134–143.

Kamp, E. M., Vermeulen, T. M. M., Smits, M. A. and Jaagsma, J. (1994): Production of Apx toxins by field strains of *Actinobacillus pleuropneumoniae* and *Actinobacillus suis*. Infect. Immun. **62,** 4063–4065.

Kokotovic, B. and Angen, O. (2007): Genetic diversity of *Actinobacillus pneumoniae* assessed by amplified fragment length polymorphism analysis. J. Clin. Microbiol. **45,** 3921–3929.

Marois-Crehan, C., Lacouture, S., Jacques, M., Fittipaldi, N., Kobisch, M. and Gottschalk, M. (2014): Development of two real-time polymerase chain reaction assays to detect *Actinobacillus pleuropneumoniae* serovars 1-9-11 and serovar 2. J. Vet. Diag. Invest. **26,** 146–149.

Marsteller, T. A. and Fenwick, B. (1999): *Actinobacillus pleuropneumoniae* disease and serology. Swine Health Prod. **7,** 161–165.

Mittal, K. R., Higgins, R. and Lariviére, S. (1983*a*): Detection of type-specific antigens in the lungs of *Haemophilus pleuropneumoniae*-infected pigs by coagglutination test. J. Clin. Microbiol. **18,** 1355–1357.

Mittal, K. R., Higgins, R. and Lariviére, S. (1983*b*): Determination of antigenic specificity and relationship among *Haemophilus pleuropneumoniae* serotypes by an indirect hemagglutination test. J. Clin. Microbiol. **17,** 787–790.

Mittal, K. R., Higgins, R. and Lariviére, S. (1987): An evaluation of agglutination and coagglutination techniques for serotyping *Haemophilus pleuropneumoniae* isolates. Am. J. Vet. Res. **48,** 219–226.

Mittal, K. R., Higgins, R. and Lariviére, S. (1988): Serologic studies of *Actinobacillus (Haemophilus) pleuropneumoniae* strains of serotype-3 and their antigenic relationships with other *A. pleuropneumoniae* serotypes in swine. J. Vet. Res. **49,** 152–155.

Molnár, É. (1990): Survey of *Actinobacillus (Haemophilus) pleuropneumoniae* infection in swine by different methods. Acta Vet. Hung. **38,** 231–238.

Nicolet, J. (1988): Taxonomy and serological identification of *Actinobacillus pleuropneumoniae*. Can. Vet. J. **29,** 578–580.

Nicolet, J., Paroz, P., Krawinkler, M. and Baumgartner, A. (1981): An enzyme-linked immunosorbent assay, using an EDTA-extracted antigen for the serology of *Haemophilus pleuropneumoniae*. Am. J. Vet. Res. **42,** 2139–2142.

Nielsen, R. (1986*a*): Serology of *Haemophilus (Actinobacillus) pleuropneumoniae* serotype 5 strains: establishment of subtypes A and B. Acta Vet. Scand. **27,** 49–58.

Nielsen, R. (1986*b*): Serological characterization of *Actinobacillus pleuropneumoniae* strains and proposal of a new serotype: serotype 12. Acta Vet. Scand. **27,** 453–455.

Nielsen, R. and O'Connor, P. J. (1984): Serological characterization of 8 *Haemophilus pleuropneumoniae* strains and proposal of a new serotype: serotype 8. Acta Vet. Scand. **25,** 96–106.

Nielsen, R., Andresen, L. O., Plambeck, T., Nielsen, J. P., Krarup, L. T. and Jorsal, S. E. (1997): Serological characterization of *Actinobacillus pleuropneumoniae* biotype 2 strains isolated from pigs in two Danish herds. Vet. Microbiol. **54,** 35–46.

Rayamajhi, N., Shin, S. J., Kang, S. G., Lee, D. Y., Ahn, J. M. and Yoo, H. S. (2005): Development and use of a multiplex polymerase chain reaction assay based on *Apx* toxin genes for genotyping of *Actinobacillus pleuropneumoniae* isolates. J. Vet. Diagn. Invest. **17,** 359–362.

Relman, D. A. (1993): Universal bacterial 16S rDNA amplification and sequencing. In: Pershing, D. H., Smith, T. F., Tenover, F. C. and White, T. J. (eds) Diagnostic Molecular Microbiology: Principles and Applications. American Society for Microbiology, Washington, D.C. pp. 489–495.

Schaller, A., Kuhn, R., Kuhnert, P., Nicolet, J., Anderson, T. J., MacInnes, J. I., Segers, R. P. A. M. and Frey, J. (1999): Characterization of *apxIVA*, a new RTX determinant of *Actinobacillus pleuropneumoniae*. Microbiology **145,** 2105–2116.

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.

Zhou, L., Jones, S. C. P., Angen, Ø., Bossé, J. T., Nash, J. H. E., 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 serovars 3, 6, and 8 *Actinobacillus pleuropneumoniae* strains. J. Clin. Microbiol. **46,** 800–803.