

ACTINOBACILLUS PLEUROPNEUMONIAE SEROTYPES IN HUNGARY

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A total of 255 *Actinobacillus pleuropneumoniae* isolates were collected from 634 lung samples representing 70 swine herds in Hungary between January 2012 and June 2016. On the basis of the indirect haemagglutination test 77 independent strains were included in the evaluation after the elimination of duplicate or multiple serotypes from the same herd. In the case of 7 herds strains of two different serotypes were identified. Fourteen Hungarian *A. pleuropneumoniae* isolates from the culture collection of the Department of Microbiology and Infectious Diseases, isolated before 2012, were also included in the evaluation (one each from 12 herds and two each from two herds, where two serotypes occurred). Out of the altogether 91 *A. pleuropneumoniae* strains 72 strains belonged to biotype I and 19 strains could be allocated to biotype II. In Hungary, the most common serotypes were serotype 2 (39.5%), 13 (15.4%), 8 (8.8%) and 16 (8.8%), but serotypes 9 (5.5%), 11 (3.3%) and 12 (3.3%) were also isolated. Twelve strains (13.2%) were untypable.

Key words: *Actinobacillus pleuropneumoniae*, distribution of serotypes, Hungary

The porcine respiratory disease complex causes serious problems and huge economic losses in the swine industry throughout the world. One of the most important respiratory agents is *Actinobacillus pleuropneumoniae*. It is responsible for haemorrhagic-necrotic pneumonia and fibrinous pleuritis in growing and fattening pigs (Chiers et al., 2010; Ózsvári and Búza, 2015). In the presence of predisposing factors such as nutritional deficiencies, management problems and sometimes bacterial and viral infections, *A. pleuropneumoniae* can cause severe acute and chronic respiratory disease. It has two biotypes: biotype I strains need

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nicotinamide adenine dinucleotide (NAD, V factor) for growth, while biotype II strains grow without NAD. The agent has important virulence factors: among others Apx toxins (RTX toxins), fimbriae, outer membrane proteins, ability of biofilm formation, presence of transporter systems and different enzymes are involved in the pathogenesis of pleuropneumonia (Chiers et al., 2010; Grasteau et al., 2011).

On the basis of surface soluble capsular polysaccharide antigens 18 serovars have been described so far (Nielsen, 1986*a,b*; Fodor et al., 1989; Nielsen et al., 1997; Blackall et al., 2002; Sárközi et al., 2015; Bossé et al., 2018). Several classical methods have been used for serotyping *A. pleuropneumoniae* strains, including slide agglutination, latex agglutination, complement fixation, indirect haemagglutination (IHA), coagglutination, ring and agar gel precipitation tests (Mittal et al., 1983*a,b*, 1987; Molnár, 1990; Nielsen et al., 1997). The IHA test is regarded to be the most specific and sensitive one among the classical serotyping methods (Mittal et al., 1983*a* and 1983*b*; Molnár, 1990; Nielsen et al., 1997). Cross reactions between serovars 1, 9 and 11, serovars 3, 6 and 8, and serovars 4 and 7 have been reported, which make serotyping rather difficult (Mittal et al., 1988; Zhou et al., 2008). Most conventional herds are infected with one or more serotypes of *A. pleuropneumoniae*, but in several cases only strains of low virulence are present (Gottschalk, 2012).

The frequency of the different serotypes and biotypes of *A. pleuropneumoniae* shows great geographical differences. Serotype 2 strains of *A. pleuropneumoniae*, which are dominant in Europe, produce two different exotoxins, while serotype 2 strains isolated in North America and Canada produce only one toxin (Gottschalk, 2012). Serotypes 2, 4 and 7 are the most common in Spain (Maldonado et al., 2009) and serotype 2 is the most frequent in Switzerland (Stärk et al., 2007). Serotypes 2, 3, 6–8, 10, 12 and 9 was reported to be dominant in the United Kingdom (Bossé et al., 2014), while serovar 15 was the leading type in Australia (Blackall et al., 2002). Serotypes 5, 7, 8 were the most frequently isolated types in Canada (Gottschalk and Lacouture, 2015). Biotype II strains are more commonly isolated in Europe, e. g. in Hungary (Fodor et al., 1989), in Denmark (Nielsen et al., 1997) and in Spain (Maldonado et al., 2009) than in the US (Perry et al., 2011).

Molecular biological methods are used more and more frequently to replace classical serotyping tests. Correlation between serovars, virulence and toxin production of the *A. pleuropneumoniae* strains described several years ago (Kamp et al., 1994; Frey et al., 1995; Marsteller and Fenwick, 1999) contributes to serotyping by detecting toxin genes; however, strains with atypical toxin production can give misleading results (Rayamajhi et al., 2005; Gottschalk, 2015). Identification of genes involved in the biosynthesis of capsular polysaccharides (Angen et al., 2008; Bossé et al., 2014; Marois-Crehan et al., 2014) and amplified fragment length polymorphism (AFLP) analysis (Kokotovic and Angen, 2007) can also be used for serotyping.

Great changes have occurred in the agricultural sector in Hungary. Earlier serotype 2 was found to be the dominant serotype in Hungary, and serotypes 1, 5, 8, 10, 11 and 12 were also detected using the coagglutination test (Molnár, 1990). However, in recent decades the international trade in the Hungarian pig industry has intensified and there is no updated publication on the frequency of *A. pleuropneumoniae* serotypes. Therefore, the aim of this study was to examine the recent distribution and frequency of the different serotypes of *A. pleuropneumoniae* in Hungary using the indirect haemagglutination test.

Materials and methods

Bacteria

A total of 255 *A. pleuropneumoniae* isolates were collected from 634 lung samples representing 70 swine herds in Hungary between January 2012 and June 2016. On the basis of the indirect haemagglutination test 77 independent strains were included in the evaluation after the elimination of duplicate or multiple serotypes from the same herd. In the case of 7 herds strains of two different serotypes were identified. Furthermore, 14 Hungarian *A. pleuropneumoniae* isolates from the culture collection of the Department of Microbiology and Infectious Diseases, isolated before 2012, were also included in the evaluation (one each from 12 herds and two each from two herds, where two serotypes occurred), making the number of total isolates 91 as reported in the following sections.

Reference strains of serovars 1–15 of *A. pleuropneumoniae* were kindly provided by Dr. Ø. Angen (Danish Veterinary Laboratory, Copenhagen), serotype 16 is an own isolate (A-85/14) (Sárközi et al., 2015), while serotypes 17 and 18 were received from J. T. Bossé (Bossé et al., 2018).

Actinobacillus pleuropneumoniae strains were isolated on Tryptone Soya Agar (TSA, Biolab Ltd., Hungary) cross-inoculated with *Staphylococcus aureus*, and cultured on chocolate agar with added 50 µg/ml NAD (Biolab Ltd., 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. The *A. pleuropneumoniae* strains were identified using standard methods (Barrow and Feltham, 2003) and 16S rRNA PCR (Relman, 1993), and stored at –80 °C until further examination.

Serological characterisation

Hyperimmune sera were produced in rabbits against *A. pleuropneumoniae* reference strains of serovars 1–18. Permission for the immunisation of rabbits was obtained from the Government Office of Pest County (PE/EA/3369-6/2016). For serotyping the indirect haemagglutination (IHA) test was performed as de-

scribed by Biberstein (1978). All 18 type strains of *A. pleuropneumoniae* were tested with all antisera. Hyperimmune sera with the highest titre were used in the examination. All field strains of *A. pleuropneumoniae* were tested with all typing sera. Only those reactions were accepted where the titre of the haemagglutination reached that of the reference strain of the serotype and the cross reactions were also typical. Reactions under 1:160 titre were neglected.

Results

The 91 Hungarian isolates included in the present study were examined in the IHA test using hyperimmune sera raised against the 18 serovars of *A. pleuropneumoniae*. Out of these 91 *A. pleuropneumoniae* strains 72 strains belonged to biotype I and 19 could be allocated to biotype II. The distribution of serotypes of *A. pleuropneumoniae* is shown in Table 1.

Table 1
Serotypes of *Actinobacillus pleuropneumoniae* in Hungary

Serotype	Before 2012		2012–2016		Total	
	strains	%	strains	%	strains	%
2	6	43	30	38.9	36	39.5
5	0	0	1	1.3	1	1.1
8	0	0	8	10.4	8	8.8
9	1	7	4	5.2	5	5.5
11	0	0	3	3.9	3	3.3
12	0	0	3	3.9	3	3.3
13	6	43	8	10.4	14	15.4
14	1	7	0	0	1	1.1
16	0	0	8	10.4	8	8.8
NT	0	0	12	15.6	12	13.2
Total	14	100	77	100	91	100

NT: non-typable

Discussion

The results of these studies indicated that serotype 2 (39.5%) and serotype 13 (15.4%) strains of *A. pleuropneumoniae* were the most frequent in Hungary. Serotype 2 is dominant in most European countries (Dubreuil et al., 2000; Chiers et al., 2002; Maldonado et al., 2009; Stärk et al., 2007), but the high frequency of serotype 13 in Hungary is unique (Fodor et al., 1989; Gottschalk et al., 2003). All our serotype 13 strains belonged to biotype II.

Untypable *A. pleuropneumoniae* strains could be isolated from clinical samples, and the frequency of the non-typable strains in Hungary is similar to the results of other authors (Eaves and Blackall, 1988; Gutiérrez et al., 1995; Klein et al., 2003; Morioka et al., 2016). Some non-typable strains could be allocated into clusters together with the serotypable ones indicating the possible loss of type-specific capsule polysaccharides (Kokotovic and Angen, 2007). Kokotovic and Angen (2007) described two clusters containing only non-typable *A. pleuropneumoniae* strains, and they supposed that these non-typable strains could represent additional serotypes. Further examinations can reveal whether our non-typable strains belong to one of these clusters.

As a result of previous serotyping using co-agglutination tests serotype 2 was dominant but serotypes 1, 3, 5, 7, 8, 10, 11 and 12 also occurred in Hungary (Molnár, 1990) and serotype 13 was described at the same time (Fodor et al., 1989). Nowadays mainly serotypes 2, 8, 16 and 13 are being isolated from clinical cases, but the isolation of non-typable strains has increased.

In conclusion, the IHA test is still one of the most sensitive classical serotyping methods. New molecular biological methods can be very helpful in serotyping *A. pleuropneumoniae* but they cannot completely replace the classical detection of antigens (Bossé et al., 2017, 2018). Serotyping is very important in the identification of *A. pleuropneumoniae*, since the haemagglutinating antigens of *A. pleuropneumoniae* are involved in the pathogenicity and in the protection against the disease caused by this agent. Regular monitoring for serotypes of *A. pleuropneumoniae* isolates is recommended because the frequency of the serotypes can change as a result of increased traffic of animals in the swine industry, thus necessitating potential adjustments in vaccine compositions.

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