

Serotype distribution of *Actinobacillus pleuropneumoniae* isolated from porcine pleuropneumonia in the Czech Republic during period 2003–2004

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ABSTRACT: A total of 245 isolates of *Actinobacillus pleuropneumoniae* included in this study were isolated from lung tissues of pigs which died of porcine pleuropneumonia. The isolates were obtained from 52 swine herds in the Czech Republic over the period 2003–2004. All the serotypes were serotyped by the coagglutination test. All isolates with cross-reactivity between serotypes 9 and 11 or 1, 9, and 11 in the coagglutination test were further serotyped by the quantitative coagglutination test. The results of serotyping showed that serotype 9 was dominant (46.5%) followed by serotypes 2 (18.5%), and 11 (14.2%). Two-way cross-reaction between serotypes 9 and 11 was found for 7.5% of the isolates. The serotypes 4, 5, 7, and 12 were found infrequently (2.4%). The isolates that could not be serotyped (11%) were classified by PCR typing system based on the *apx* and *omlA* genes into the serotype groups 1, 9, 11 (7.5%); 2, 8 (2.3%), and 7, 13 (1.1%).

Keywords: pig; epidemiology; serotyping; quantitative coagglutination; polymerase chain reaction

Actinobacillus pleuropneumoniae is the etiological agent of a highly contagious respiratory disease of pigs designated as porcine pleuropneumonia. The disease can be in acute, peracute, subacute, or chronic form and is characterized by fibrinous pleuritis and hemorrhagic pneumonia (Sebunya and Saunders, 1983).

A. pleuropneumoniae can be categorized on the basis of nicotinamide adenine dinucleotide (NAD) requirement for growth into two biovars: NAD dependent biovar 1 and NAD independent biovar 2 (Pohl et al., 1983). Recently, an integration of the serotyping schemes for biovars 1 and 2 was proposed (Nielsen et al., 1997). Within these biotypes, individual serotypes are differentiated on the basis of capsular polysaccharides and lipopolysaccharides (Nielsen, 1990). A total of 15 serotypes of *A. pleuropneumoniae* (1 to 15) have been published, in addition to subtypes like 5A, 5B, K2:O7 and K1:O7 (Nicolet, 1992; Nielsen et al., 1996, 1997; Gottschalk et al., 2000; Blackall et al., 2002).

Several serological techniques have been developed for serotyping of *A. pleuropneumoniae* such as slide agglutination, tube agglutination, ring precipitation, coagglutination, immunodiffusion, indirect hemagglutination and counterimmunoelectrophoresis (Mittal et al., 1992). Of those, the coagglutination test is considered to be a sensitive and specific method for serotyping of *A. pleuropneumoniae* (Mittal et al., 1992, 1993a). Molnar and Molnar (1994) also mentioned the coagglutination test to be a sensitive, reproducible and rapid serotyping method although cross-reactions had been observed between some of the serotypes. Monoclonal antibodies were used for demonstration of common epitopes located on O antigen of lipopolysaccharide that are responsible for cross-reactions between the serotypes 1, 9, and 11 (Barbosa et al., 1996), and between serotypes 4 and 7 (Lebrun et al., 1999). Cross-reactivity observed between the serotypes 3, 6 and 8 was attributed to group-specific antigens which are independent of

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serotype specificity. Mittal et al. (1988) suggested that their localization is in cell-wall components and described quantification of the type and group-specific antigens for differentiation of serotype 3, 6 and 8 by quantitative coagglutination test. Mittal et al. (1993b) also used quantitative coagglutination test for identification of isolates with cross-reactivity between serotypes 1, 9, and 11. Although, monoclonal antibodies targeted to specific epitopes were considered to be more precise for serological classification (Lacouture et al., 1997), production and characterization of specific monoclonal antibodies for serotypes 9 and 11 has not yet been reported.

Because of serological cross-reactions, alternative methods for precise identification of individual serotypes were also tested. Individual serotypes have been distinguished into five groups on the basis of production the Apx exotoxins or on the gene-toxin profile (Frey et al., 1993; Beck et al., 1994). A PCR identification system of *A. pleuropneumoniae* has been proposed on the basis of presence or absence of *apx* genes in the genome of individual serotypes (Frey et al., 1995; Gram et al., 2000). The *Apx1VA* gene presented in all *A. pleuropneumoniae* serotypes has also been used in discrimination of *A. pleuropneumoniae* by PCR-REA technique (Jaglic et al., 2004). The *omlA* gene has also been shown as a suitable target for molecular typing by PCR or PCR-REA (Gram et al., 2000; Cho and Chae, 2003).

Porcine pleuropneumonia is a respiratory disease resulting in high morbidity and mortality of pigs and accordingly economic losses within the swine industry worldwide, including the Czech Republic. Determination of the distribution of *A. pleuropneumoniae* serotypes is essential in the control, therapeutic or immunoprophylactic programmes, in order to facilitate elimination of the disease. Therefore the objective of our study was determination of the serotype distribution of *A. pleuropneumoniae* from lung tissues of pigs which died from porcine pleuropneumonia in the Czech Republic during the period 2003–2004.

MATERIAL AND METHODS

Bacterial strains and culture media

Twelve reference strains of *A. pleuropneumoniae* (strains 4074, S1536, S1421, M62, K17, Femo,

WF83, 405, CVJ13261, D13039, 56153, 8329, serotypes 1–12, respectively) were selected for the preparation of hyperimmune rabbit sera or were used as positive control for serotyping and molecular typing.

A total of 254 isolates of *A. pleuropneumoniae* have been included in this study. These strains originated from 52 farms located in the Czech Republic and were isolated from pathological lesions in pulmonary tissues of pigs which died from porcine pleuropneumonia in 2003–2004.

The lung tissues were inoculated onto blood agar plates (Blood Agar Base No.2, HiMedia, India) with a streak of *Staphylococcus aureus* for production of NAD. The isolates were incubated at 37°C for 18 h. After another passage through PPLO agar (PPLO Agar Base, HiMedia, India) supplemented with NAD, the cultures were suspended in phosphate buffered saline solution (PBS) of pH 7.2 and bovine fetal serum and stored at –70°C until used.

Preparation of hyperimmune sera

New Zealand white rabbits were inoculated with formalinized whole cell suspensions of the reference strains. Antigen preparation and immunization were performed as described by Mittal et al. (1982). Sera were stored at –20°C until used.

Coagglutination (CoA) test

The preparation of coagglutination reagents, processing and evaluation were described earlier in details by Mittal et al. (1983). Whole – cell suspensions were prepared according to the method of Mittal et al. (1993b) with slight modifications. Bacterial cultures were harvested in PBS containing 0.3% formalin and then adjusted to an optical density (OD) of 0.5 at 550 nm. The specificity of the CoA technique was verified using antigens of the reference strains. Cell suspension of *Staphylococcus aureus* Cowan I (CCM 2352) in PBS and in normal rabbit serum without antibodies to *A. pleuropneumoniae* was used as negative controls.

Quantitative coagglutination (QCoA) test

Preparation of absorbed hyperimmune sera, as well as the procedure and evaluation of the test

were carried out according to Mittal et al. (1993b) with small modifications. Bacterial culture was harvested in PBS containing 0.3% formalin, adjusted to OD 1 at 550 nm, and the antigen was further two-fold diluted in PBS from 1 : 5 to 1 : 80. Negative controls were used as described in CoA.

PCR identification of *apx* and *omlA* genes

A loopful of a bacterial colony was resuspended in 100 µl of deionized water, boiled for 10–15 min and centrifuged at 10 000 g for 10 min. The supernatant was used for the PCR reaction. Preparation of the reaction mixture, PCR, detection and visualization of the amplification products were performed according to the procedure by Gram et al. (2000). DNA obtained from the reference strains *A. pleuropneumoniae* was used as a positive control; deionized water was used as a negative control.

RESULTS AND DISCUSSION

Serotyping of the isolates by CoA revealed high percentage of cross-reactions between serotypes 9 and 11 (54.7%) or 1, 9, and 11 (8.7%). In total, 11.0% of the isolates were non-typable by CoA (Table 1). By non-typable isolates we understand isolates that react with none or with two or more of the coagglutination reagents for *A. pleuropneumoniae* serotypes 1 to 12, but exclude those isolates that showed cross-reactivity with coagglutination reagents for serotypes 9 and 11 or 1, 9, and 11. Cross-reactivity observed between the serotypes 1, 9, and 11 is most likely associated with the presence of common immunodominant epitopes located on the O antigen of LPS (Barbosa et al., 1996).

The sensitivity of QCoA for differentiation of serotypes 1, 9, and 11 was tested using the refer-

Table 1. Serotyping of *A. pleuropneumoniae* isolates by CoA test

Serotype	No. positive	% positive
1	0	0
2	47	18.5
3	0	0
4	1	0.4
5	1	0.4
6	0	0
7	2	0.8
8	0	0
9	0	0
10	0	0
11	12	4.7
12	2	0.8
9/11 ^a	139	54.7
1/9/11 ^b	22	8.7
NT ^c	28	11.0
Total	254	100

^aisolates with cross-reactivity

^bisolates with cross-reactivity

^cnon-typable isolates that react with none or with two or more of the coagglutination reagents for *A. pleuropneumoniae* serotypes 1 to 12, but exclude those isolates that showed cross-reactivity with coagglutination reagents for serotypes 9 and 11 or 1, 9, and 11

ence strains *A. pleuropneumoniae* of serotypes 1, 9, and 11 (Table 2). The antigens of the serotypes 1 and 9 showed reactivity with homologous coagglutination reagents but showed no reactivity with heterologous coagglutination reagents. Antigen of the serotype 11 reacted with a homologous coag-

Table 2. Serotyping of *A. pleuropneumoniae* reference strains by QCoA test

Reference strain (serotype)	Test antigen	Titer of antigen detected by QCoA with rabbit antisera against serotype:			Serotype identification
		1	9	11	
40741 (1)	WC	80	0	0	1
CVJ13261 (9)	WC	0	40	0	9
56153 (11)	WC	0	5	40	11

WC formalinised whole-cell suspension

Table 3. Serotyping of *A. pleuropneumoniae* isolates by QCoA test

Serotype	No. positive	Titer of antigen detected by QCoA with rabbit antisera against serotype		
		1	9	11
1	0	0	0	0
9	118	0	20–80	0–5
11	24	0–1	0–5	10–40
9/11 ^a	19	0–1	10–40	10–40

^aisolates with the cross-reactivity between serotypes 9 and 11

glutination reagent at titre 40 and heterologous coagglutination reagent for serotype 9 at titre 5. The results obtained confirm the ability of QCoA to differentiate between the reference strains of serotype 1, 9, and 11 by determining the titres of antigens following reaction with homologous and heterologous coagglutination reagents.

The isolates showing cross-reactivity between serotypes 9 and 11 (139 isolates) or 1, 9, and 11 (22 isolates) examined by CoA were more precisely tested by QCoA. Determination of the titres of antigens resulted in classification of 118 isolates into serotype 9 and 24 isolates into serotype 11. Identical titres of antigen to coagglutination reagents for serotype 9 and 11 were detected in only 19 isolates which were classified as isolates with cross-reactivity between serotypes 9 and 11 (Table 3). The isolates classified by QCoA into the serotype 9 or 11 either did not react with the heterologous coagglutination reagent or reacted only with a concentrated antigen or at antigen dilution of 1 : 5. At higher dilutions of antigen, cross-reactions were eliminated while positive reactions with homologous coagglutination reagents could be seen. The results obtained are consistent with the

fact that strains of serotype 9 and 11 may also be antigenically diverse and show a varying degree of cross-reactivity with polyclonal rabbit sera (Mittal et al., 1993b).

Twenty eight isolates which were finally signed as non-typable in the CoA test has not been further serotyped by the QCoA for specific differential susceptibility of this test used in our study. These isolates were classified by PCR according to Gram et al. (2000) into the *apx/omlA* pattern 1 (serotype 1, 9, and 11; 19 isolates), pattern 2 (serotypes 2, 8; 6 isolates) and pattern 7 (serotypes 7, 13; 3 isolates) (Table 4). A precise serotype determination of isolates belonging to the highly virulent *apx/omlA* of pattern 1 has been problematic also using genetic methods (in particular the serotypes 9 and 11). Two studies reported on successful differentiation of these two serotypes. Hennessy et al. (1993) used arbitrarily primed PCR to distinguish these two serotypes and de la Puente-Redondo et al. (2000) reported on variability of *tbpA* gene in serotypes 9 and 11. However, arbitrarily primed PCR is known to be quite difficult to standardise (Black, 1993) and concerning the *tbpA* gene variability, we previously amplified the gene sequence and digested the PCR product with restriction endonuclease *AsnI* exactly as described (de la Puente-Redondo et al., 2000).

By combining the results from CoA and QCoA, the serotype distribution among the Czech isolates could be determined. Serotype 9 was found to have the highest prevalence (46.5%) followed by serotype 2 (18.5%), 11 (14.2%), and isolates showing two-way cross-reaction between serotypes 9 and 11 (7.5%). The occurrence of other serotypes (4, 5, 7 and 12) was sporadic (in a total 2.4%). Serologically non-typable isolates were examined by PCR and classified as serotypes 1, 9, 11 (7.5%); 2, 8 (2.3%) and 7, 13, (1.1%).

The results suggest that there has been a change in the serotype distribution in the Czech Republic. Skolova and Gois (1987) found that serotype 2 had

Table 4. Differentiation of *A. pleuropneumoniae* non-typable isolates in the CoA by PCR typing of the *apx* and *omlA* genes

<i>apx/omlA</i> pattern	<i>apxI</i>	<i>apxII</i>	<i>apxIII</i>	<i>omlAI</i>	<i>omlAII</i>	<i>omlAIII</i>	<i>omlAIV</i>	<i>omlAV</i>	Serotype	No. positive
1	+	+	–	+	–	–	–	–	1, 9, 11	19
2	–	+	+	–	+	–	–	–	2, 8	6
7	–	+	–	–	–	+	–	–	7,13	3
Total										28

the highest prevalence, while Satran and Nedbalcova (2002) reported that serotype 9 had the highest frequency followed by serotype 2. The results of serotyping in our study showed that serotype 9 was dominant followed by serotypes 2 and 11. We also found an increased frequency of serotype 11 (from 4.1% to 14.2%) compared to the previous study by Satran and Nedbalcova (2002). Only a single isolate was found of serotype 5, a strongly virulent serotype, which is dominant in Canada, in the USA and Italy (Dubreuil, 2000).

Distribution of *A. pleuropneumoniae* serotypes determined in our study differ from the dominant serotypes reported by Dubreuil et al. (2000) in other European countries (serotypes 2 in Denmark, Sweden, Norway and Switzerland; 2, 3 and 8 in the United Kingdom; 9 in France; 2, 7 and 9 in Germany; 2, 3 and 7 in Hungary; 3 in Ireland and Belgium; 2, 9 and 11 in the Netherlands; 1 and 9 in Poland; 2, 4 and 7 in Spain; 2 and 9 in Croatia). This indicates that there is a risk of introducing new serological varieties of *A. pleuropneumoniae* by purchasing pigs from regions with other distribution of serotypes.

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