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Genotype Profiles of *Actinobacillus pleuropneumoniae* Isolated from Pigs in Vojvodina, Serbia

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ABSTRACT

***Background*:** *Actinobacillus pleuropneumoniae* (*A. pleuropneumoniae*) is one of the most important bacterial respiratory pathogens. It is the only etiological agent of porcine pleuropneumonia (PPP) or it appears as a secondary bacterial infection in the swine respiratory disease complex (PRDC). In Serbia, apart from the identification of serotype 2, no tests have been performed to establish the presence of other *A. pleuropneumoniae* serotypes in the pig population. The aim of this study was to perform genotyping of *A. pleuropneumoniae* isolates originating from pig farms in Serbia by apx genes and using multiplex polymerase chain reaction (mPCR).

***Materials, Methods & Results*:** Isolates of *A. pleuropneumoniae* examined in this study were obtained from lungs with macroscopically visible alterations characteristic of a *A. pleuropneumoniae*. A total of 46 isolates were examined. They were extracted from the lung tissue samples of pig carcasses from 9 farms across different parts of Serbia. Genotyping of isolates was performed in the previously described manner. Briefly, 5 pairs of oligonucleotide primers were used for amplification of 4 different *apx* genes which encode synthesis of exotoxins (ApxI , ApxII , ApxIII i ApxIV) characteristic for all *A. pleuropneumoniae* serotypes and biovars. Amplification of appropriate genome parts was performed with a reaction chain polymerase (PCR) in multiplex (m) format using appropriate diagnostic kits to extract DNA from bacteria and perform mPCR reaction. The results of genotyping of 46 isolates of *A. pleuropneumoniae* indicate the existence of a large number of different serotypes of *A. pleuropneumoniae* on the studied farms or that different serotypes of this microorganism circulate in the pig population in Serbia. In addition to the detection of dominant serotype 2, which was established on 7 farms, of which in 4 farms it was the only detected serotype, in the examined pig population the presence of serotypes 3, 5, 6, 7 and 9 was also found. Furthermore, the presence of 2 different serotypes of *A. Pleuropneumoniae* was also detected on 3 farms; on the first farm serotypes 2 and 3, on the second farm serotypes 2 and 6, and on the third farm serotypes 2 and 7.

***Discussion*:** Although the research was done with a relatively small number of isolates of *A. pleuropneumoniae*, comparing the obtained results with the results on the presence and prevalence of appropriate serotypes from other countries, we concluded that there is significant diversity of this pathogen in the pig population in farms of Serbia. Detection of different serotypes of *A. pleuropneumoniae* in the pig population and the presence of several different serotypes on 1 farm was established for the very first time in Serbia. All isolates from our study can be characterized as highly virulent, considering that the clinical symptoms, pathological findings and the results of bacteriological examination indicated *A. pleuropneumoniae* to be the cause of animal death. Like in the neighbouring countries, the strongly pathogenic serotype 9 and the less pathogenic serotype 2 are the most frequently identified causative agents of porcine pleuropneumonia in the Autonomous Province of Vojvodina, Republic of Serbia. The necessity to establish the presence of all *A. pleuropneumoniae* serotypes in the pig population, and in particular to determine the presence of different serotypes on individual farms, is crucial for several reasons: making a definitive diagnosis; development of prophylactic strategies for medicines; implementation of immunoprophylactic vaccination.

Keywords: swine, porcine pleuropneumonia, PPP, serovar 2, serotype, lung, PCR.

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INTRODUCTION

Porcine pleuropneumonia (PPP) caused by *Actinobacillus pleuropneumoniae* is a bacterial infection of the respiratory system of peracute, acute or chronic course that most often occurs in growing pigs (weaned piglets, pre-fattening pigs) and fatteners. It grows on blood culture media, creates beta hemolysis and shows CAMP phenomenon. Isolates of *A. pleuropneumoniae* can be classified into 2 biovars depending on the need for nicotinamide dinucleotide phosphate (NADP) for growth, with biovar 1 being NADP-dependent and significantly larger biovar 2 being NADP-independent [4]. Based on the differences in the structure of lipopolysaccharides (LPS) of the bacterial wall and capsular polysaccharides, 16 serovars of this bacterium have been described recently [2,7]. *Actinobacillus pleuropneumoniae* produces protein cytotoxins ApxI, ApxII, ApxIII and ApxIV which belong to the so-called pore-forming RTX family of toxins, which are secreted by different serotypes of *A. pleuropneumoniae* in different combinations, via a type-1 secretory mechanism [9]. The most important virulence factors of *A. pleuropneumoniae* are capsular polysaccharides, LPS (endotoxin), outer membrane proteins and exotoxins (Apx - toxins) [3]. Apx - toxins are directly involved in the creation of clinical symptoms and differ among themselves in hemolytic and cytotoxic activity [9].

The aim of this study was to perform genotyping of *A. pleuropneumoniae* isolates originating from pig farms in Serbia by apx genes and using multiplex polymerase chain reaction (mPCR).

MATERIALS AND METHODS

Sampling and laboratory analysis

Actinobacillus pleuropneumoniae isolates were recovered from the lung tissue samples of died pigs, with tipical lesions on the lungs (elevated cherry-colored red areas/red areas of hemorrhagic-necrotic pneumonia), originating from 9 farms amongst different regions in Serbia. Tissue samples were plated on Columbia agar with 5% defibrinated ovine blood[1]. *Staphylococcus aureus* culture[2] as the source of the V factor, to facilitate the isolation of *A. pleuropneumoniae* was used. Subsequent cultivation of bacteria was performed on a chocolate agar supplemented with 10 mg/L NAD[2]. All bacterial cultivations were carried out at 37°C and 5% CO_2.

A total of 46 isolates were genotyped in the previously described manner [6]. Briefly, 5 pairs of oligonucleotide primers were used for amplification of 4 different *apx* genes which encode synthesis of exotoxins (ApxI, ApxII, ApxIII i ApxIV) characteristic for all *A. pleuropneumoniae* serotypes and biovars. Amplification of appropriate genome parts was performed with a reaction chain polymerase (PCR) in multiplex (m) format using appropriate diagnostic kits[3] to extract DNA from bacteria and perform mPCR reaction.

Separation of amplified PCR products was performed by horizontal electrophoresis in 1.5% agarose gel with the addition of ethidium bromide and the presence of electrophoretic buffer for 35 min 115V and 85A. Visualization and positioning of amplified PCR products was performed by observation under UV light, that is by comparing the position of PCR products with standard lengths of individual DNA fragments (from 100 to 3,000 bp) originating from molecular DNA rulers.

Amplification product form or size of the 4 RTX toxin genes was used for genotyping of *A. pleuropneumoniae* isolates (Table 1).

RESULTS

The results of genotyping of 46 isolates of *A. pleuropneumoniae* are shown in Table 2. The presented results indicate the existence of a large number of different serotypes of *A. pleuropneumoniae* on the studied farms or that different serotypes of this microorganism circulate in the pig population in our country. The largest number of isolates belongs to serotype 2 (28/46), which was established on 7 farms, in 2 farms as the only serotype, and 3 farms in co-infection with isolates typified as serotype 7, serotype 3 and serotype 6, respectively.

DISCUSSION

Serotypes 2 (28 isolates), 9 (6 isolates) 5 (4 isolates), and 6 (4 isolates) were the most common, only 2 isolates belonged to serotype 7 and serotype 3. This finding is not surprising given that the high prevalence of this serotype of *A. pleuropneumoniae* has been established earlier in Serbia [5]. Unfortunately, a more detailed characterization of *A. pleuropneumoniae* isolates has never been performed in Serbia, which is why there is no more precise information regarding this causative agent, including data on serotyping or the prevalence of appropriate serotypes circulating in the pig population.

Table 1. PCR amplification of ApxI, ApxII, ApxIII and ApxIV genes of all 15 serotypes of *Actinobacillus pleuropneumoniae* isolated from lungs of dead pigs from farms in Serbia.

Serotype	ApxIA	ApxIB	ApxII	ApxIII	ApxIV	ApxIV	ApxIV	ApxIV
	723bp	811bp	965bp	396bp	1600bp	2000bp	2400bp	2800bp
1	+	+	+				+	
2		+	+	+				+
3			+	+			+	
4		+	+	+	+			
5a	+	+	+					+
5b	+	+	+					+
6		+	+	+		+		
7		+	+					+
8		+	+	+				+
9	+	+	+		+			
10	+	+						+
11	+	+	+		+			
12		+	+				+	
13		+	+				+	
14	+	+					+	
15		+	+	+				+

Table 2. Genotyping results of 46 isolates *Actinobacillus pleuropneumoniae* from lung tissue samples originating from dead pigs isolates from 9 farms in Serbia.

Farm	Serotype (n of isolates)	Established *apx* toxins
I	5ab (4)	ApxIA, ApxIB, ApxII
II	2 (3)	ApxIB, ApxII, ApxIII
	3 (2)	ApxII, ApxIII
III	9/11 (6)	ApxIA, ApxIB, ApxII
IV	2(6)	ApxIB, ApxII, ApxIII
	6 (4)	ApxIB, ApxII, ApxIII
V	2 (4)	ApxIB, ApxII, ApxIII
VI	2 (3)	ApxIB, ApxII, ApxIII
VII	2 (4)	ApxIB, ApxII, ApxIII
	7 (2)	ApxIB, ApxII
VIII	2 (3)	ApxIB, ApxII, ApxIII
IX	2 (5)	ApxIB, ApxII, ApxIII

Researchers from different EU countries report results on the presence and prevalence of the corresponding *A. pleuropneumoniae* serotypes. Thus, serotypes 2, 5, 6, 9 and 11 were established in the Netherlands [1], serotypes 2, 3, 5 and 6 in Denmark, serotypes 2, 3, 5 and 9 in Belgium, and serotypes 2 and 9 in Germany [3]. In our study, in addition to the detection of dominant serotype 2, serotypes 3, 5, 6, 7 and serotype 9 were also identified. Although the study was conducted on a relatively small number of farms or with a smaller number of *A. pleuropneumoniae* isolates, comparing the results with other countries it can be concluded that there is a significant diversity of this microorganism. All isolates from our study can be characterized as highly virulent, considering that the clinical symptoms, pathological findings and the results of bacteriological examination indicated *A. pleuropneumoniae* to be the cause of animal death.

An interesting finding was a virulent serotype 9 found on a farm that uses imported piglets for fattening purpose (imports from Hungary). Since the farm produces only fatteners, an episode of acute *A. pleuropneumoniae* caused by this serotype was most likely of autogenous origin, in other words the disease appeared in animals that are carriers, which is why we assumed that this serotype was most likely introduced from Hungary. Also on 3 farms the presence of 2 different serotypes of *A. pleuropneumoniae* was found, on 1 serotype 2 and serotype 7, on the other serotype 2 and serotype 3, and on the third serotype 2 and serotype 6. This finding indicates that different strains of *A. pleuropneumoniae* can be present on the farm, which was established for the first time in Serbia. Co-infection with different serotypes of *A. pleuropneumoniae* has several implications: first, not all strains are necessarily pathogenic; second, if strains have been identified as pathogenic, due to the fact that different *A. pleuropneumoniae* serotypes produce different RTX toxins that generate clinical symptoms, their effects can be summarized making the clinical picture and pathological findings more complex; third, when it comes to diagnosis, especially if the intent is to get isolates of *A. pleuropneumoniae*, material for laboratory testing, most often altered lungs, should be sampled from different production or age categories, since one serotype may be dominant in one and another in different category of pigs on the farm; fourth, during the implementation of the immunoprophylaxis (vaccination) program, it is necessary to determine all the serotypes of *A. pleuropneumoniae* present on the farm, this is especially important if the plan is to introduce immunoprophylactic vaccination with bacterin and/or mixed vaccines. Finally, despite the characteristic clinical picture and pathological findings, today, as in other infectious diseases, the isolation and serotyping of *A. pleuropneumoniae* isolates is the standard in the diagnosis.

Actinobacillus pleuropneumoniae causes significant economic losses as this bacterium may be the sole cause of pneumonia. It does not need the cooperation of other infectious agents to manifest its pathogenic effect, which is why it is one of the primary respiratory pathogens. Also, infected animals are characterized by lifelong germination (primarily in the tonsils), and in the pig breeding system as practiced on most of farms in Serbia, which involves continuous production (from "piglets to fatteners") in one place ("one roof"), there are almost ideal conditions for spread and long-term persistence in the pig population. This is supported by the fact that the prevalence of *A. pleuropneumoniae* as an independent cause of swine pneumonia in Serbia was found in the percentage of 21 to 30% [8,10], and in the complex of respiratory disease of pigs (PRDC) in the percentage as high as 56% [8]. All this indicates the importance of this etiologic agent in the etiopathogenesis of swine pneumonia.

CONCLUSION

It can be concluded that, like in the neighbouring countries, the strongly pathogenic serotype 9 and the less pathogenic serotype 2 are the most frequently identified causative agents of porcine pleuropneumonia in the Autonomous Province of Vojvodina, Republic of Serbia. Sporadic findings of other serotypes of *A. pleuropneumoniae*, apparently introduced by imported animals, emphasise the necessity of exact correct diagnostics including serotype classification apx gene profile determination for correct assessment of the epizootological situation.

MANUFACTURERS

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[3]Thermo Fisher Scientific Baltics. Vilnius, Lithuania.

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REFERENCES

1 **Angen O., Heegaard P.M., Lavritsen D.T. & Sørensen V. 2001.** Isolation of *Actinobacillus pleuropneumoniae* serotype 2 by immunomagnetic separation. *Veterinary Microbiology*. 79: 19-29.

2 **Bosse J.T., Li Y., Sarkozi, R., Gottschalk M., Angen Ø., Nedbalcova K. & Langford P.R. 2017.** A unique capsule locus in the newly designated *Actinobacillus pleuropneumoniae* serovar 16 and development of a diagnostic PCR. *Journal of Clinical Microbiology*. 55: 902-907.

3 **Dubreuil J.D., Jacques Mario., Khyali R.M. & Gottschalk M. 2000.** *Actinobacillus pleuropneumoniae* surface polysaccharides: their role in diagnosis andimmunogenicity. *Animal Health Research Reviews*. 1(2): 73-93.

4 **Gottschalk M. 2015.** The challenge of detecting herds sub-clinically infected with *Actinobacillus pleuropneumoniae*. *Veterinary Journal*. 206: 30-38.

5 **Ivetić V., Žutić M., Romanić S., Valter D. & Drezga J. 1996.** Prilog poznavanju aktinobacilusne pleuropneumonije i serovarne distribucije kauzalnog agensa u velikim aglomeracijama svinja. *Zbornik kratkih sadržaja. 9. Savetovanja Veterinara Srbije* (Zlatibor, Serbia).pp.107-109.

6 **Rayamajhi N., Sung J., Sang G.K., Deog Y.L., Jeong M. & Han S.Y. 2005.** Development and use of a multiplex polymerase chain reaction assay based on Apx toxin genes for genotyping of *Actinobacillus pleuropneumoniae* isolates. *Journal of Veterinary Diagnostic Investigation*. 17: 359-362.

7 **Sarkozi R., Makrai L. & Fodor L. 2015.** Identification of a proposed new serovar of *Actinobacillus pleuropneumoniae*: Serovar 16. *Acta Veterinaria Hungarica*. 63: 444-450.

8 **Savić B., Radanović O., Jovičić D., Nešić K., Ivanović S., Stevančević O., Cvetojević Đ. & Kasagić D. 2015.** Survey of infectious agents associated with porcine respiratory disease complex (PRDC) in serbian swine herds using polymerase chain reaction (PCR) detection. *Acta Veterinaria Belgrade*. 65(1): 79-88.

9 **Schaller A., Kuhnert P., de la Puente-Redondo V.A., Nicolet J. & Frey J. 2000.** Apx toxins in Pasteurellaceae species from animals. *Veterinary Microbiology*. 74: 365-376.

10 **Žutić M., Lepšanović Z., Krnjajić D. & Ašanin R. 1999.** Actimicrobial susceptibility and plasmid profiles of *Actinobacillus pleuropneumoniae* strains isolated from swine. *Book of Abstracts 13th International Congress of the Hungarian Society for Microbiology* (Budapest, Hungary). p.114.