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**Comparative examination of *Actinobacillus pleuropneumoniae* strains
isolated from swine**

Ph.D. thesis

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INTRODUCTION

Large scale husbandry dominates the swine industry in many countries. Porcine respiratory disease complex (PRDC) can cause huge economic losses in these units due to the predisposing effects of the intensive technology. *Actinobacillus pleuropneumoniae* plays a crucial role in the aetiology of PRDC. It is facultative pathogen and present on the mucous membrane of the respiratory tract of swine and can cause disease in the case of different predisposing factors. Only swine is susceptible to *A. pleuropneumoniae*, clinical signs can mainly be seen in 12-16-week-old pigs. Acute haemorrhagic-necrotic pneumonia and fibrinous pleuritis are typical in growing pigs, while chronic lesions can be seen in the lungs at the slaughterhouse.

On the basis of nicotinamide adenine dinucleotide (NAD) requirement to growth, *A. pleuropneumoniae* biotype I (NAD-dependent) and biotype II (NAD-independent) can be distinguished and the strains can be assigned into 16 serotypes. Mainly biotype I strains were isolated in Hungary in the last 30 years. The pathological and histological lesions caused by the two biotypes cannot be differentiated. *A. pleuropneumoniae* strains are able to produce four toxins (ApxI-II-III-IV), out of them ApxIV toxin is produced by all *A. pleuropneumoniae* serotypes but only in the infected host. Detection of antibodies against ApxIV confirms the infection of the pigs. Animals showing acute signs of the disease are treated with beta-lactam, fluoroquinolone, pleuromutilin, macrolide and tetracycline antibiotics. In order to prevent the disease bacterin, toxoid and serotype specific autogenous vaccines are applied.

The aim of our study was characterisation of freshly isolated *A. pleuropneumoniae* strains and strains from the collection of the Department of Microbiology and Infectious Diseases.

- *Examination of serotypes in Hungary*

Serotyping of field strains and strains from the strain collection was planned using indirect haemagglutination test (IHA) and polymerase chain reaction (PCR) based on the detection of toxin genes. We wanted to examine the distribution of the *A. pleuropneumoniae* serotypes isolated from lung and tonsil samples collected in different swine herds.

- *Identification of strains on species level*

Identification of strains was proposed using Biolog Microstation™ ID System which is based on utilization of carbon sources by *A. pleuropneumoniae* strains. The aim of this examination was testing the system whether it can be used for the identification of *A. pleuropneumoniae* in the diagnostic work.

Analysis of whole genome macrorestriction pattern of *A. pleuropneumoniae* strains was also planned.

- *Antibiotic susceptibility tests*

Examination of antimicrobial sensitivity of *A. pleuropneumoniae* isolates using disc diffusion technique and determination of minimum inhibition concentration (MIC) values was intended in order to analyse the change in antibiotic susceptibility and to find correlation between the results gained with different methods.

- *Preparation of selective media*

Selective culture methods were developed for the isolation of *A. pleuropneumoniae* from tonsils of wild boars and domestic pigs.

MATERIALS AND METHODS

Origin of samples

Bacterial cultures were made from 634 swine lungs collected from 70 herds representing different parts of Hungary. A total of 40 tonsils from swine and 68 ones from wild boars were also examined. The 15 serotype reference strains and 75 *A. pleuropneumoniae* strains isolated between 1995 and 2010 and deposited in the strain collection of Department of Microbiology and Infectious Diseases were included in the examinations, too.

Identification of *A. pleuropneumoniae*

NAD dependence, haemolysis, biochemical and morphological features of strains were examined using standard bacteriological methods.

Utilization of 95 carbon sources of 68 field strains and 6 reference strains was characterised with the Biolog Microstation™ ID System (Biolog Inc, California, USA).

Genetic relationship of *A. pleuropneumoniae* strains was analysed using pulse field gel electrophoresis (PFGE).

Serotyping of *A. pleuropneumoniae* strains, antibiotic susceptibility tests

Serotyping of the strains was done using the indirect haemagglutination test (IHA) and frequency of the serotypes was compared with previous results. Hyperimmune sera were produced against 15 reference strains and 6 field strains which were untypable. All reference strains of *A. pleuropneumoniae* were tested with all hyperimmune sera.

Serotypes of 60 *A. pleuropneumoniae* strains were defined by detecting toxin genes with PCR. Whole genome sequencing of strains of a proposed new serotype was carried out with Microbes NG Sequencing Facility and serotype specific primers were prepared.

Antibiotic resistance of 60 *A. pleuropneumoniae* strains was tested using the disc diffusion and broth dilution methods with 20 antibiotics. Genes were detected which are responsible for tetracycline and beta-lactam resistance.

Preparation of selective media

A basic medium was prepared to selective media. Susceptibility of 15 *A. pleuropneumoniae* strains to 90 antibiotics was examined using the disc diffusion method. Then susceptibility of 6 Gram-negative bacterium species isolated from the upper respiratory tract of swine to the best 25 antibiotics was tested using the same method. MIC of lincomycin, polymyxin-B and vancomycin against some Gram-negative and Gram-positive bacteria was determined.

Selective medium "A", the reference one, contained bacitracin, lincomycin, crystal violet and nystatin, while in medium "B" vancomycin, polymyxin-B, cycloheximid and crystal violet were the inhibitory agents. Efficacy of selective media was tested by Gram-negative and Gram-positive bacteria isolated from upper respiratory tract of swine which were inoculated on the media. Media were inoculated with samples from tonsils of wild boars and swine.

RESULTS

Distribution of *A. pleuropneumoniae* in Hungary

Total of 255 *A. pleuropneumoniae* strains were isolated from swine. The animals were generally 12-16-week-old or older and showed clinical signs of acute pleuropneumonia before they died. In some cases similar clinical signs and post mortem lesions were seen in young piglets around weaning age and the aetiological role of *A. pleuropneumoniae* in these cases was described by us. Distribution of serotypes in Hungary is shown in Table 1.

Table 1: Distribution of serotypes in Hungary

Serotypes	Before 2012		2012-2016		Total	
	strain	%	strain	%	strain	%
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
UT	0	0	12	15,6	12	13,2
Total	14	100	77	100	91	100

UT: untypable

The results of serotyping using PCR for the detection of toxine genes and IHA are summarised in Table 2 showing correlation between the two methods.

Table 2: Comparison of serotyping using IHA and PCR for the detection of toxin genes

Serotypes (PCR)		Serotypes (IHA)						
	Number	2	8	9	12	13	16	UT
1	2			2				
2/8/15	22	14	8					
5	11						9	2
7	3	1	1					1
9/11	1			1				
12/13	11				2	9		
UT	10	8						2
Total	60	23	9	3	2	9	9	5
%	100	38	15	5	3,5	15	15	8,5

UT: untypable

A total of 56 strains proved to be untypable using the IHA test. Hyperimmune sera were produced against six of them, five strains could be serotyped and they were described as a new serovar, serovar 16 by us recently. Whole genome sequencing of the reference strain of serotype 16 also proved that it is a new serotype. Based on the difference in the *cps* locus specific primers were designed. The sixth examined strain can represent an other new serotype.

Identification of strains and antibiotic susceptibility tests

Thirty strains were identified as *A. pleuropneumoniae* using the Biolog system, whereas in the case of 40 strains *A. lignieresii* was on the first place on the identification list. Altogether 44.8% of the strains were identified as *A. pleuropneumoniae* by the system and the strains were able to metabolize 20 different carbon sources. The system is not able to distinguish between biotypes and serotypes.

All strains were typable with *Apal* restriction endonuclease. Strains were classified into 11 clusters. *A. pleuropneumoniae* serotype 2 strains proved to be highly diverse, while strains of biotype 2 were really closely related. Serotype 9, 16 and the untypable strains formed separated clusters.

On the basis of MIC of the different antibacterials most strains were susceptible to beta-lactams, fluoroquinolones, phenicols, macrolides and tiamulin. Forty-fifty % of the strains were resistant against gentamicin, spectinomycin, penicillin, erythromycin, oxytetracycline and sulfamethoxazole-trimethoprim (SXT). All strains were resistant against sulfamethoxazole. Examination of the antibiotic susceptibility of *A. pleuropneumoniae* with the disc diffusion method proved to be less sensitive, so the results of the disc diffusion test have to be interpreted carefully.

There were no beta-lactam resistance genes detected, but tetracycline resistance genes were harboured by 16 strains.

Preparing selective media and examination of tonsils from wild boars

The most effective basic medium was prepared from Mueller-Hinton agar with the addition of 0.3 mg/ml NAD, 5% sheep blood and 0.01 g/ml yeast extract. *A. pleuropneumoniae* was cultured on both of selective media („A” and „B”), *A. pleuropneumoniae* caused strong beta-haemolysis on the selective media. Anyway *A. pleuropneumoniae* was isolated from tonsils on medium „A” only. From 40

swine tonsils 3 *A. pleuropneumoniae* strains were isolated, but we didn't isolate it from tonsils of culled sows.

One *A. pleuropneumoniae* strain was isolated on medium "A" out of 68 tonsils of wild boars. The strain belonged to serotype 12 and its MIC profile was similar to that of *A. pleuropneumoniae* strains isolated from domesticated pigs. It was susceptible to beta-lactams, enrofloxacin, tiamulin, doxycycline and phenicols, resistant against penicillin, aminoglycosides, oxytetracycline, thulatromycin and tilmicosin.

DISCUSSION

Distribution of *A. pleuropneumoniae* in Hungary

The sampled animals were generally 12-16-week-old or older and showed clinical signs of acute pleuropneumonia before they died. In some cases similar clinical signs and post mortem lesions were seen in young piglets around weaning age and the aetiological role of serotype 11 of *A. pleuropneumoniae* was described. This herd was infected with an other serotype of *A. pleuropneumoniae* and there was maternal immunity only against that serotype. We thought that the herd became recently infected with serotype 11, and in the absence of maternal immunity against serotype 11 typical acute respiratory clinical and pathological signs appeared in very young piglets. This case confirmed the serotype specific protection.

Chronic pleuropneumonia lesions were present upon slaughterhouse examination, there were no clinical signs, and the animals did not die in this form. Reduced daily weight gain and reduced feed conversion cause the economic loss. According to our results the Hungarian conventional swine herds are infected with one or more serotypes of *A. pleuropneumoniae*. Altogether 89% of field strains needed NAD for requirement, 11% of them could grow without it, this result is similar to the European data. Biotype II strains are more common in Europe, especially in Hungary, than in other countries but the reason for it is not known.

There was no change in the frequency of serotype 2 and 9. These serotypes are also dominant in those countries which export pigs to us. Serotype 8 and 16 are new ones in Hungary, serotype 16 has been isolated only in Hungary yet.

There is close correlation between serotypes and toxin genes. *ApxIA*, *apxB*, *apxII* and *apxIV* genes are harboured by serotype 16 strains like 5 serotypes. Using only PCR method for serotyping based on detection of toxin genes can give false result, but serotyping based on detection of capsule polysaccharides and lipopolysaccharides identified a new serotype. On the basis of detection of toxin genes two strains were serotyped as serotype 5 with PCR as well but neither serotyping with IHA nor the macrorestriction pattern confirmed it. These strains do not belong to serotype 16, they can be members of a still not described serotype.

Identification of strains, PFGE examination, antibiotic susceptibility tests

Biolog system can be moderately used for identification on species level of *A. pleuropneumoniae* strains, because Biolog database includes the utilization of carbon sources only of a few field strains. Metabolic profile based on carbon source utilization of *A. pleuropneumoniae* and *A. lignieresii* is really similar. These two species are closely related which is confirmed by the similarity of the 16S rRNS gene sequences, too. A detailed morphological, cultural and biochemical examination of the strains can prevent misidentification. Based on our examinations a new database including more strains is recommended.

Almost half of the strains (44%) were of serotype 2 in PFGE examination. Serotype 2 strains in Europe are highly virulent, produce two kinds of toxin (ApxII and ApxIII). Nine clusters were determined by the analytic program, so serotype 2 strains proved to be highly diverse. Serotype 13 strains are sporadic, they formed 2 subclusters when examined with PFGE, and they proved to be really closely related. A similar close genetic relationship was detected in the case of serotype 9 and 16 strains, they formed each one group.

Susceptibility of *A. pleuropneumoniae* strains to the most commonly used antibiotics in swine industry was examined. In the case of some antibiotics results of the disc diffusion and the broth dilution tests were slightly different but the strains showed susceptibility to most antibiotics using both tests. Significant difference between the two methods was seen when penicillin, tilmicosin, thulatromycin, SXT and tiamulin were tested. Examination of the antibiotic susceptibility of *A. pleuropneumoniae* with the disc diffusion method proved to be less sensitive, so the results of the disc diffusion test have to be interpreted carefully. The exact susceptibility of the strains to antibiotics can be measured only with the broth dilution method. The antibiotic susceptibility of the strains isolated in different years decreased like in other countries in Europe.

Presence of not all resistance genes could be examined, however not only resistance genes are responsible for phenotypic resistance, but other mechanism, as well. So even in the absence of known resistance genes bacteria can show resistance against certain antibiotics.

Efficacy of selective media

The basic blood agar for selective media can be used in the diagnostic work, serotyping and DNA-extraction.

Gram-positive bacteria were inhibited by vancomycin in medium "B". Huge amount of members of Enterobacteriaceae family can be found in tonsils so polymyxin-B was used to inhibit them. As a result of MIC determination of polymyxin-B Enterobacteria were more sensitive than *A. pleuropneumoniae*. After inoculation *A. pleuropneumoniae* strains grew and caused haemolysis on both selective media, but the bacterium was isolated on medium „A" only. Based on the culturing results selective media can be used to isolate *A. pleuropneumoniae*.

Characterisation of *A. pleuropneumoniae* strain isolated from tonsil of a wild boar

Seropositivity of wild boars to *A. pleuropneumoniae* was examined in several countries, showing that the agent is carried by European and North-American wild boars but clinical signs and lesions have not been described yet.

We isolated a serotype 12 strain of *A. pleuropneumoniae* for the first time from a wild boar on selective medium. It was supposed that the isolated strain would be more susceptible to antibiotics than strains from domesticated pigs, but the result was really similar to domesticated pigs' antibiotic resistance profile. It allows the supposition that the strain came from domestic pigs.

NEW SCIENTIFIC RESULTS

1. We isolated serotype 16 of *A. pleuropneumoniae* for the first time and characterised the toxin gene patterns of the strains.
2. We determined the frequency of serotypes in Hungary, and compared the results of serotyping based on the detection of toxin genes with PCR and indirect haemagglutination test.
3. We confirmed that *A. pleuropneumoniae* can be cause acute disease in one-month-old piglets, too.
4. We confirmed that Biolog Microstation™ ID System is suitable to examine utilization of carbon sources of *A. pleuropneumoniae* strains, but it can be used for identification on species level only with limitation.
5. PFGE was proved to useful for classifying *A. pleuropneumoniae* strains, serotypes, and it can be used to epidemiological survey. As a result of PFGE examination *A. pleuropneumoniae* strains formed 11 clusters.
6. We determined antibiotic resistance of 60 *A. pleuropneumoniae* strains isolated from different herds with 20 antibiotics.
7. We constructed a basic medium to selective media and isolated *A. pleuropneumoniae* bacterium species on selective media from tonsils of swine.
8. We isolated and characterised an *A. pleuropneumoniae* strain from tonsil of wild boar.

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