Antimicrobial susceptibility of *Actinobacillus pleuropneumoniae* strains isolated from pig's clinical samples in Serbia

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Summary

Porcine pleuropneumonia, caused by *Actinobacillus pleuropneumoniae* is one of the most important bacterial diseases of the pig respiratory tract. One hundred and forty eight *Actinobacillus pleuropneumoniae* strains isolated from pig's clinical samples were tested for their susceptibility to 11 antimicrobials by disk diffusion method. Isolates were obtained from the pathoanatomically altered pig's lung that died with signs of respiratory disease. Antimicrobial discs with following concentration were used: ampicillin 10 µg, amoxicillin 10 µg, amoxicillin/clavulanic acid 20/10 µg, cefotaxime 30 µg, gentamicin 10 µg, penicillin 10 IU, streptomycin 10 µg, tetracycline 30 µg, enrofloxacin 5 µg, lincospectin 15/200 µg, florfenicol 30 µg and trimethoprim/sulfamethoxazole 1.25/23.75 µg. Isolated strains showed various susceptibility to tested antimicrobials. Seventy-five percent of isolates were resistant to one or more drugs frequently used in the treatment of pig's diseases at farms in Serbia. All 148 isolates were susceptible to cefotaxime, enrofloxacin and florfenicol, while 96% of them were susceptible to amoxicillin/clavulanic acid and lincospectin. Susceptibility of isolates to amoxicillin, gentamicin and trimethoprim/sulfamethoxazole were 91%, 77% and 64%, respectively. The highest resistance rates were determined for tetracycline and streptomycin, 34% and 31%, respectively. When compared to our previous results, we noticed a decrease in resistance to tetracyclines and trimethoprim/sulfamethoxazole. Our results indicate that *A. pleuropneumoniae* strains isolated from pig's clinical samples in Serbia show high susceptibility to antimicrobial agents which are most often used in the pig industry.

Keywords: *Actinobacillus pleuropneumoniae*, antimicrobial susceptibility, pig

Porcine pleuropneumonia, caused by *Actinobacillus pleuropneumoniae* (*A. pleuropneumoniae*), is one of the most important bacterial diseases of the pig respiratory tract. This highly contagious respiratory disease is widely spread among pig-keeping countries causing major economic losses in the swine industry (21, 30). The most important *A. pleuropneumoniae* virulence factors include surface polysaccharides (18), iron uptake systems (4), Apx toxins (11, 16, 28), components of anaerobic metabolism (17), and outer membrane proteins (8, 12). On the basis of the β -nicotinamide adenine dinucleotide (β -NAD) requirement for growth *A. pleuropneumoniae* has been classified into biovar 1 (β -NAD dependent) and biovar 2 (β -NAD independent). Biovar 2 can synthesize NAD in the presence of specific pyridine nucleotides or their

precursors (6, 21). Each biovar is further divided into serovars. Fifteen serovars of both biovars *A. pleuropneumoniae* based on capsular and lipopolysaccharide antigens have been described, with a variation in virulence and regional distributions. There are 13 serotypes of biotype 1, and 2 serotypes of biotype 2. Certain strains of biovar 2 have the same antigenic determinants as serovars 2, 4, 7 and 9 of biovar 1 (5, 21). The four major RTX toxins ApxI, ApxII, ApxIII and ApxIV are cytolytic and/or hemolytic, and secreted by the different serotypes in various combinations (11). Compared to ApxI, ApxII and ApxIII, ApxIV is produced by all serotypes of *A. pleuropneumoniae*, highly specific to *A. pleuropneumoniae* and expressed only *in vivo* but not *in vitro* conditions (28). Most of the pathological consequences of porcine pleuropneu-

monia can be attributed to these Apx toxins (16). The role of biofilms in the pathogenicity of *A. pleuropneumoniae* is also very important (29). Cells of *A. pleuropneumoniae* in biofilm were 100 to 30,000 times more resistant to antimicrobials than their planktonic counterparts (3). The disease is transmitted by aerosol within short distances or by direct contact with infected pigs. Clinical signs are fever, severe respiratory distress with cyanosis, coughing and epistaxis. Characteristic pathoanatomic alterations are fibrinohemorrhagic necrotizing broncho-pneumonia and fibrinous pleuritis (13). Growing pigs are most likely to be affected when they are 12-16 weeks old, but the disease can occur in all ages of swine. Efficient antimicrobial therapy is an important protective measure for the control and prevention of porcine pleuropneumonia (3, 20). A broad range of antimicrobial agents used for the treatment of disease including β -lactams (penicillin, amoxicillin, ampicillin, and ceftiofur), fluoroquinolones, gentamicin, trimethoprim/sulfamethoxazole, tiamulin, lincomycin/spectinomycin, and tetracyclines (25, 30). Isolates of *A. pleuropneumoniae* are generally reported to be susceptible toward fluoroquinolones, ceftiofur and florfenicol, but various antimicrobial resistance patterns have been observed to others antimicrobials in different countries (10, 32).

The last research of the antimicrobial susceptibility of *A. pleuropneumoniae* in Serbia was published seven years ago (34), but the current situation is unknown. Therefore, the aim of our study was to investigate the antimicrobial susceptibility of *A. pleuropneumoniae* strains isolated from clinical samples originating from pig breeding farms in Serbia in order to provide adequate treatment of infected animals and thus prevent the spread of disease in swine population.

Material and methods

Bacterial strains. *A. pleuropneumoniae* isolates were obtained from the pathoanatomically altered pig's lung that died with signs of respiratory disease. A total of 148 clinical isolates were tested during the period from 2014-2015 (2014 – 89, 2015 – 59) from 9 large pig breeding farms located in northwest Serbia. Samples of pig lungs were inoculated on 5% sheep blood agar with *Staphylococcus aureus* as a source of NAD, and incubated in a jar with reduced oxygen and enhanced carbon dioxide content. Suspicious colonies were subcultivated on chocolate agar with PolyVitek (bioMérieux, France) and sheep blood agar, as before. *A. pleuropneumoniae* were identified on the basis of the Gram-stains, haemolysis on 5% sheep blood agar, positive CAMP test, requirement for NAD and urease production.

Antimicrobial susceptibility testing. Susceptibility to antimicrobials of *A. pleuropneumoniae* isolates was determined by the disk diffusion method using chocolate Mueller-Hinton agar (Mueller Hinton II agar, HiMedia, India) according to CLSI recommendation (8). The diameter of the inhibition zone of the individual drug was determined after 20-22 hours of incubation at 37°C. Isolates were classified as susceptible, intermediate or resistant according to the CLSI and manufacturer's instructions. *A. pleuropneumoniae* ATCC 27090 was used as a quality control strain. Antimicrobial discs with following concentration were used: ampicillin 10 µg, amoxicillin 10 µg, amoxicillin/clavulanic acid 20/10 µg, cefotaxime 30 µg, gentamicin 10 µg, penicillin 10 IU, streptomycin 10 µg, tetracycline 30 µg, enrofloxacin 5 µg, lincospectin 15/200 µg, florfenicol 30 µg and trimethoprim/sulfamethoxazole 1.25/23.75 µg (Rosco Diagnostica, Denmark; Bioanalyse, Turkey).

Results and discussion

A. pleuropneumoniae clinical strains isolated in our study showed various susceptibility to the tested antimicrobials. The results are shown in Tab. 1.

Seventy-five percent of isolates were resistant to one or more of the antimicrobials frequently used in the treatment of pig's diseases at farms in Serbia. All 148 isolates were susceptible to cefotaxime, enrofloxacin and florfenicol, while 96% of them were susceptible to amoxicillin/clavulanic acid and lincospectin. Similar results were obtained in other studies for florfenicol (14, 32), ceftiofur/cefotaxime (19) and enrofloxacin (24). A high susceptibility to lincospectin correlates with the observed susceptibility of 95% in neighboring Croatia (15), but differs from the results obtained in Taiwan where only 2.4% of the 211 investigated strains were susceptible to this antibiotic (31). High rates of susceptibility of 91% were observed for ampicillin and amoxicillin, which is in agreement with the reports by Matter and Kucerova (19, 22). On the other hand, these results differ from the results of Vanni (30) who found 69% and 82% resistance to ampicillin and amoxicillin, respectively. The susceptibility to gentamicin in our study was 77%, which is much higher than susceptibil-

Tab. 1. Antimicrobial susceptibility of 148 isolates of *A. pleuropneumoniae*

Antibiotics	Susceptible		Intermediate		Resistant	
	No. isolates	%	No. isolates	%	No. isolates	%
Ampicillin	136	91.82	2	1.35	10	6.75
Amoxicillin	136	91.82	0	0	12	8.10
Amoxicillin/clavulanic acid	143	96.82	0	0	5	3.37
Cefotaxime	148	100.00	0	0	0	0
Gentamicin	114	77.02	17	11.48	17	11.48
Streptomycin	93	62.83	9	6.08	46	31.08
Enrofloxacin	148	100.00	0	0	0	0
Lincospectin	143	96.62	0	0	5	3.37
Tetracycline	89	63.13	8	5.40	51	34.45
Florfenicol	148	100.00	0	0	0	0
Trimethoprim/sulfamethoxazole	95	64.18	17	11.48	36	24.32

ity rates of 5.7% and 43% published by other authors (26, 31). In our study the highest resistance rates were determined to tetracycline (34%) and streptomycin (31%). A significantly higher level of resistance to tetracycline was observed in Spain 85% (14) as well as in Australia 75% (9). Higher levels of resistance of 88.4% and 90.7% for chlortetracycline and oxitetracycline, respectively, were identified in Canada (17). When compared with our previous results (34), we observed a significant decrease in the tetracycline resistance from 53% to 34% as well as in trimethoprim/sulfamethoxazole resistance from 56% to 24%. Similar results for sulfonamides were registered in the Czech Republic (25). A significant reduction of resistance for these two antimicrobial agents might have occurred as a result of their reduced usage during recent years, especially in treating animals from the category 15-40 kg of body weight. In several previous Serbian studies (27, 33, 35) it was found that the most common respiratory bacterial pathogens in pigs were *Pasteurella multocida*, *A. pleuropneumoniae* and *Haemophilus parasuis*. Generally, in the treatment of infections caused by these pathogens tetracycline and trimethoprim/sulfamethoxazole are less efficient in comparison to other antibiotics, so this may partly explain the reduced usage of these two drugs in veterinary practice in Serbia. Of the total of 148 *A. pleuropneumoniae* tested isolates, 112 (75%) showed resistance to one or more drugs. Seventy two isolates showed resistance to one drug, 24 to two, while 16 strains were resistant to three or more drugs. Among the 72 isolates, 34 were resistant to tetracycline, 19 to streptomycin, 14 to trimethoprim/sulfamethoxazole, 4 to ampicillin and one isolate was resistant to gentamicin. Patterns of co-resistance to the two drugs were as follows: 9 isolates were resistant to streptomycin and trimethoprim/sulfamethoxazole, 6 to gentamicin and tetracycline, 6 to gentamicin and streptomycin, one to ampicillin and amoxicillin, one to amoxicillin and trimethoprim/sulfamethoxazole, while one was resistant to amoxicillin/clavulanic acid and gentamicin. In this study, multidrug resistance (MDR) was considered as resistance to three or more antimicrobials. A total of 16 isolates showed multidrug resistance pattern (Tab. 2).

Tab. 2. Multidrug resistant (MDR) patterns detected in *A. pleuropneumoniae* clinical isolates

MDR pattern	Number of isolates
Str, Tet, T/S	6
Amp, Amx, Tet	3
Gen, Str, Lsp	3
Amx, A/C, Str, Lsp, T/S	1
Amx, A/C, Gen, Str, Lsp, T/S	1
Amp, Amx, Gen, Str, Tet, T/S	2

Explanations: Str – streptomycin; Tet – tetracycline; T/S – trimethoprim/sulfamethoxazole; Amp – ampicillin; Amx – amoxicillin; Gen – gentamicin; Lsp – lincospectin; A/C – amoxicillin/clavulanic acid

It is interesting, to note that all five strains resistant to lincospectin belonged to the multiresistant groups, as well as 6 of the 12 strains resistant to amoxicillin. Multidrug resistance of *A. pleuropneumoniae* strains has also been registered in other regions of the world. For example, in Canada five isolates demonstrated multidrug resistance to penicillins, streptomycin, tetracyclines and sulfanomides, whereas three others showed multiresistance to streptomycin, sulfonamides and tetracyclines (17). In many countries different levels resistance of *A. pleuropneumoniae* (10, 14, 25, 30) have been determined. Bacterial pathogens that cause diseases in pigs including *A. pleuropneumoniae* often demonstrate resistance to drugs commonly used in veterinary practice. Without knowledge of local resistance, use of antimicrobials creates a risk for treatment failure, but also for the spread of resistance among *A. pleuropneumoniae* isolates and the emergence of an unfavorable epizootic situation. Antimicrobial resistance is one of the major problems of human and veterinary medicine of the 21st century. Continuous monitoring of antimicrobial resistance and responsible usage of drugs in livestock are very important in protecting against bacterial pathogens (23). Classes of antimicrobials such as cephalosporins and fluoroquinolones are relevant for human health and their use in livestock production should be limited or avoided as far as possible (1). Furthermore, antimicrobial usage in animals increases the possibility of acquiring resistance determinates in target bacteria as well as in commensal and zoonotic bacteria (2). There are numerous examples indicating that the level of bacterial resistance to antimicrobials depends of their consumption, and that consumption sooner or later leads to the development of resistance.

The results of the study showed high susceptibility rates of *A. pleuropneumoniae* clinical isolates to cefotaxime, enrofloxacin, florfenicol, amoxicillin/clavulanic acid, lincospectin, ampicillin and amoxicillin. The highest resistance rates were established for tetracycline and streptomycin. It is important to emphasize that we noticed a decreasing trend in resistance to tetracycline and trimethoprim/sulfamethoxazole. Continuous monitoring of antimicrobial susceptibility of *A. pleuropneumoniae*, as well as other respiratory bacterial pathogens, is important for prompt and adequate treatment of infected animals and prevention of the spread of resistance and reduces their current level.

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