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ANTIMICROBIAL RESISTANCE OF *ACTINOBACILLUS PLEUROPNEUMONIAE* FIELD ISOLATES IN AUTONOMOUS PROVINCE OF VOJVODINA, REPUBLIC OF SERBIA

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Abstract

Actinobacillus pluropneumoniae (APP) is one of the most important bacterial respiratory pathogens in swine. It is the only etiological agent of porcine pleuropneumoniae (PPP) or appears as a secondary bacterial infection of the swine's respiratory disease complex (PRDC). Antibiotics are still the most effective measure for reducing mortality and severity of clinical symptoms in most parts of the world. Nevertheless, in recent years, resistance to certain antibiotics such as tetracycline, penicillin, aminoglycosides and others has been increasing. The aim of this study was to examine the resistance of *A. pleuropneumoniae* isolates in the area of AP Vojvodina. The samples were collected from dead pigs' lung tissue from 14 farms. Bacterial strains were isolated on agar with 5% sheep blood and *Staphylococcus aureus* culture as a source of factor V and for subculture chocolate agar enriched with 10 mg/L NAD was included as well. After the examination of morphological and cultural characteristics, biochemical identification was performed using standard bacteriological tests. APP was confirmed by PCR. A total of 35 bacterial isolates were tested on antimicrobial resistance using the disc diffusion method with 11 antibiotics. Antibiotics from the group: aminoglycosides, sulfonamides, fluoroquinolones, phenicols, tetracyclines and beta-lactam antibiotics were used. The isolates collected in the area of AP Vojvodina, from 2015 to 2022, retained high sensitivity to florfenicol, cephalosporins and fluoroquinolones. However, increasing

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antibiotic resistance was observed for penicillin, tetracycline and all tested aminoglycosides.

Key words: *A. pleuropneumoniae*, antibiotics, resistance, swine

ANTIMIKROBNA REZISTENCIJA IZOLATA *ACTINOBACILLUS PLEUROPNEUMONIAE* NA TERITORIJI AUTONOMNE POKRAJINE VOJVODINE, REPUBLIKA SRBIJA

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Kratak sadržaj

Actinobacillus pleuropneumoniae (APP) se ubraja u najznačajnije bakterijske respiratorne patogene svinja. Jedini je etiološki agens *porcine pleuropneumoniae* (PPP) ili se pojavljuje kao sekundarna bakterijska infekcija u kompleksu respiratorne bolesti svinja (PRDC). Antibiotici su i dalje najefikasnija mera za smanjenje mortaliteta i težine kliničke slike u većini dijelova svijeta, međutim tokom prethodnih godina javlja sve češća pojava rezistencije na pojedine antibiotske preparate poput tetraciklina, penicilina, aminoglikozida i dr. Cilj ove studije jeste ispitati rezisteniciju izolata *A. pleuropneumoniae* na prostoru AP Vojvodine. Uzorci su prikupljeni iz plućnog tkiva uginulih svinja sa 14 farmi. Za izolaciju *A. pleuropneumoniae* korišten je agar sa 5% ovčije krvi i kultura soja *Staphylococcus aureus* kao izvor V faktora, a za presejavanje čokoladni agar obogaćen sa 10 mg/L NAD. Nakon ispitivanja morfoloških i kulturelnih karakteristika izvršena je biohemijska identifikacija primenom standardnih bakterioloških testova. APP je potvrđen pomoću PCR metode. U ispitivanju antibiotske osetljivosti 35 bakterijskih izolata korištena je disk difuziona metoda sa ukupno 11 antibiotika. Korišteni su antibiotici iz grupe:, aminoglikozida, sulfonamida, fluorohinolona, fenikola, tetraciklina i beta-laktamskih antibiotika AB preparata. Izolati prikupljeni na teritoriji AP Vojvodine od 2015 do 2022 godine zadržali su visoku osetljivost na florfenikol, cefalosporine i fluorohinolone. Kod drugih antimikrobnih sredstava kao što su penicilin,

tetraciklin i svi testirani aminoglikozidi, uočena je povećana rezistencija na antibiotike.

Ključne reči: *A. Pleuropneumoniae*, antibiotici, rezistencija, svinje

INTRODUCTION

Swine pleuropneumonia is one of the most important swine bacterial respiratory pathogens occurring worldwide. The occurrence of acute epidemics characterized by high mortality results in economic damage, loss in production, reduced growth and high medical costs. Many herds get infected with several strains. High virulence strains can be present in a herd for a long period of time without clinical symptoms or lesions in the slaughterhouse.

The causative agent of the disease, *Actinobacillus pleuropneumoniae* (*A. pleuropneumoniae*) is a small, Gram-negative, encapsulated rod with typical coccobacillary morphology. *A. pleuropneumoniae* isolates can be classified into two biovars depending on the need for nicotinamide dinucleotide phosphate (NADP) for growth, with biovar 1 dependent on NADP and significantly less common biovar 2 being independent of NADP (Gottschalk, 2012). There are currently 19 recognized serovars of *A. pleuropneumoniae*, based on their capsule synthesis genes (cps), with certain differences in geographical distribution and pathogenicity (Bossé et al., 2018; Sassu et al., 2018). In most European countries, serovar 2 is a dominant strain causing most disease outbreaks (Sárközi et al., 2018). This was confirmed recently in a German study based on more than 200 APP isolates originating from the same geographical area from the years 2010–2019, where 64% were found to belong to serovar 2 as the predominant serotype (Schuwerk et al., 2021). The most important virulence factors of *A. pleuropneumoniae* are capsular polysaccharides, LPS (endotoxin), and outer membrane proteins and exotoxins (Apx - toxins). *A. pleuropneumoniae* produces protein cytotoxins ApxI, ApxII, ApxIII and ApxIV belonging to the so-called RTX toxin family, which secrete different serotypes in various combinations, through type 1 secretory mechanism (Schaller et al., 2000).

Antimicrobial resistance is becoming a major problem both in Serbia and worldwide. The aim of this study is to examine the susceptibility of pathogens to antibiotics that are most commonly used in the treatment of this disease. Infections in humans caused by antimicrobial resistance bacteria of animal's origin is becoming an urgent threat to the control of bacterial infections. Identification of antibiotic-resistant or susceptible strains is essential in the fight

against antibiotic-resistant pathogens. Determination of susceptibility to antimicrobial drugs is performed by disk diffusion method or by determining the minimum inhibitory concentration (MIC).

MATERIAL AND METHODS

The isolates used in this study were from lung tissue of pigs' carcasses from 14 different farms in the territory of AP Vojvodina (Figure 1). Lung tissue samples were collected from carcasses with typical lesions on the lungs (elevated cherry-coloured red areas/red areas of haemorrhagic-necrotic pneumonia) with pronounced interlobular edema and covered with fibrin deposits.

Figure 1. Distribution of *A. pleuropneumoniae* isolates in the territory of AP Vojvodina (The map was downloaded from https://www.superjoden.nl/regioni-srbije-mapa.html)

A. pleuropneumoniae isolates were plated on Columbia Blood Agar with 5% defibrinated sheep blood. Culture of *Staphylococcus aureus* strain was used as a source of factor V and for subculture chocolate agar with PolyVitex (BioMerieux, Marcy l'Etoile, France) enriched with 10 mg/L NAD was included as well. Incubation lasted for 24 h at 37 °C in the presence of 5% CO_2. After examination of morphological and cultural characteristics, biochemical identification was performed using standard bacteriological tests (Žutić et al., 2009).

APP was also confirmed by PCR. Five pairs of oligonucleotide primers described by Rayamajhi et al., 2005, are used for the amplification of 4 "Apx" genes encoding exotoxin synthesis ("ApxI", "ApxII", "ApxIII" and "ApxIV") characteristic of all serotypes and biovars of *A. pleuropneumoniae*. Amplification of the appropriate parts of the genome are performed using the polymerase chain reaction (PCR) in multiplex format (mPCR) using the set for performing the mPCR reaction (EURx). A "primer-mix" is prepared for a total reaction volume of 50 µl, with an oligonucleotide-primer concentration of 0.5 µM and a maximum number of oligonucleotide-primer pairs of 5 per one reaction according to the following formula: 25 µl master mix + 25 µl (primer mix + sample).

Urease activity and positive Christie-Atkins-Munch-Peterson (CAMP) test further refer to *A. pleuropneumoniae*. From fresh clinical samples, 70% of the strains were 'sticky' and difficult to remove from the plate, which correlates with their ability to form a biofilm in vitro.

A total of 85 isolates were isolated from 14 farms across AP Vojvodina (Figure 1). Thirty-five isolates were tested for their susceptibility to a panel of antimicrobials by disk diffusion method on Mueller Hinton Agar Plate (MH agar) with the addition of 5% defibrinated horse blood for fastidious bacteria. The following antibiotics were used: penicillin G (6 mg), amoxicillin (25 mg), ampicillin (10 mg), ceftiofur (30 mg), trimethoprim/sulfamethoxazole (1.25 mg / 23.75 mg), enrofloxacin (5 mg), florfenicol (30 mg), gentamicin (15 mg), neomycin (30 IU), streptomycin (10 IU), tetracycline (30 IU). The isolates were classified as S-sensitive, I-intermediate, and R-resistant. In the assessment of resistance, intermediate isolates were considered resistant. Interpretation of growth inhibition followed the clinical breakpoints approved by the Clinical Laboratory Standards Institute (CLSI 2018).

RESULTS AND DISCUSSION

Altogether, 51.4% of isolates were susceptible to tetracyclines. Less than half of the isolates, more precisely, 48.6% were sensitive to penicillin. The ef-

ficacy results within the aminoglycoside group differ markedly. Only 28.6% of isolates were sensitive to streptomycin and 34.3% to neomycin. Gentamicin, on the other hand, shows effectiveness in 65.7% of isolates (Table 1.). Our results show an identical efficiency of 74.3% and a low rate of resistance to fluoroquinolone (enrofloxacin) and sulpha drug. With 80% of sensitive isolates, 3rd generation cephalosporin was in the second place in terms of efficacy. Florfenicol shows best results and the lowest rates of antimicrobial resistance. Only 17.1% of isolates were resistant (including intermediates).

Table 1. Resistance of 35 *A. pleuropneumoniae* isolates found in the territory of AP Vojvodina

Antibiotic	S– sensitive (%)	I–intermediate (%)	R–resistant (%)
Penicillin G	48.6	0	51.4
Ampicillin	68.6	2.8	28.6
Amoxicillin	68.6	5.7	25.7
Tetracycline	51.4	8.6	40
Ceftiofur	80	8,6	11.4
Gentamicin	65.7	5.7	28.6
Neomycin	34.3	17.1	48.6
Streptomycin	28.6	17.1	54.3
Trimethoprim/sulfamethoxazole	74.3	0	25.7
Enrofloxacin	74.3	11.4	14.3
Florfenicol	82.9	5.7	11.4

Antimicrobials are intensively used in swine production for therapeutic, metaphylactic and prophylactic purposes, which has led to an increased risk of bacterial resistance over time (Jensen et al., 2006; Aarestrup et al., 2008). Moreover, the movement of pigs between herds or between countries is another key factor that can contribute to the spread of antimicrobial-resistant isolates in swine populations.

Antimicrobials that are widely used in the treatment of swine pleuropneumonia were tested. Penicillin, tetracycline, amoxicillin, cephalosporin and florfenicol are recommended by the latest guidelines for pleuropneumonia therapy.

As already reported, β-lactams have shown high levels in vitro activity against *A. pleuropneumoniae* (Yoshimura et al. 2002; Matter et al., 2007). However, in recent years, a relatively large number of resistant isolates have

been reported, mainly penicillin, while amoxicillin and ampicillin are slightly effective. Our results confirm this rising trend mostly for penicillin. Minor antimicrobial activity of aminoglycosides against *A. pleuropneumoniae* isolates in our study are confirmed by the results in other studies such as Gutierrez-Martin (2006) and Matter et al. (2007). Tetracyclines and sulpha drugs are still among the recommended antimicrobial agents for the treatment of swine pleuropneumonia (Burch et al., 2008). However, these antimicrobials have been used extensively to treat several swine diseases over decades, so increased resistance rates have been reported in several European countries (Gutierrez-Martın et al., 2006; Hendriksen et al., 2008). Cephalosporins (3rd generation) have shown very good results. Florfenicol provides the best results and the lowest rates of antimicrobial resistance. In vitro activity of florfenicol in clinical isolates *A. pleuropneumoniae* has been extensively studied and low rates of resistance have been found in Germany, South Korea, Spain, and Japan (Gutierrez-Martın et al. 2006, Morioka et al., 2008). The fluorinated chloramphenicol derivative is a broad-spectrum antimicrobial drug, which has been licensed in Europe since 2000 for the treatment of bacterial respiratory tract infections in pigs (Kehrenberg et al., 2004).

CONCLUSION

Isolates collected from the territory of AP Vojvodina from 2015 to 2022 retained high sensitivity to certain antimicrobial drugs used in therapy of *A. pleuropneumoniae* such as florfenicol, cephalosporin and fluoroquinolones. With other antimicrobials such as penicillin, tetracycline and aminoglycosides, increasing antibiotic resistance is observed. This confirms the importance of continuous monitoring of clinical isolates. In order for therapy to be successful and the use of antibiotics rational, it is necessary to have reliable data on the antimicrobial resistance for any pathogenic microorganisms. All farms should perform an antibiogram test before treating pleuropneumonia. It is an additional cost, but it pays off in the eventually. The therapy will be more successful, and the occurrence of antibiotics resistance will be reduced.

For years, the cause of the disease and the disease itself have been studied both together and separately. The acquired knowledge has enabled the improvement of diagnostic tests, vaccines and relatively effective eradication strategies. However, the *A. pleuropneumoniae* remains a significant cause of large economic losses for the swine industry and there is more space for the improvement of control and eradication of this pathogen.

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Author's Contribution:

KK and OS made contributions to the idea of the publication, organisation of work and writing the manuscript; OS reviewed the manuscript; BS gave the final approval of the manuscript to be published.

Competing interest

The authors declare that they have no conflict of interest. The authors alone are responsible for the content and writing of the paper.

REFERENCES

1. Aarestrup F.M., Duran C.O., Burch D.G.S. 2008. Antimicrobial resistance in swine production. Animal health research reviews, 9, 2, 135–148. doi: 10.1017/S1466252308001503.
2. Bossé J.T., Li Y., Sárközi R., Fodor L., Lacouture S., Gottschalk M., Amoribieta M.C., Angen Ø., Nedbalcova K., Holden M.T.G., Maskell D.J., Tucker A.W., Wren B.W., Rycroft A.N., Langford P.R. 2018. Proposal of serovars 17 and 18 of *Actinobacillus pleuropneumoniae* based on serological and genotypic analysis. Veterinary Microbiology, 217, 1–6. doi: 10.1016/j.vetmic.2018.02.019.
3. Burch D.G.S., Duran, C.O., Aarestrup F.M. 2008. Guidelines for antimicrobial use in swine. *In* Guide to Antimicrobial Use in Animals. Eds. L. Guardabassi, L.B. Jensen, H. Kruse, 10th edition, Blackwell Publishing, Oxford, 102–125.
4. CLSI. 2018. Performance Standards for Antimicrobial Susceptibility Testing. CLSI Document M100, Wayne, PA, Clinical and Laboratory Standards Institute.
5. CLSI. 2018. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals. 4th edition, CLSI Supplement VET08, Wayne, PA, Clinical and Laboratory Standards Institute.

6. Gottschalk M. 2012. Actinobacillosis. *In* Diseases of swine. Eds. J.J. Zimmerman, L.A. Karriker, A. Ramirez, K.J. Schwartz, G.W. Stevenson 10th edition, Wiley-Blackwell, 653–669.
7. Gutierrez-Martın C.B., Garcıa del Blanco N., Blanco M., Navas J., Rodrıguez-Ferri E.F. 2006. Changes in antimicrobial susceptibility of Actinobacillus pleuropneumoniae isolated from pigs in Spain during the last decade. Veterinary microbiology, 115, (1-3), 218–222. doi: 10.1016/j.vetmic.2005.12.014.
8. Hendriksen R.S., Mevius D.J., Schroeter A., Teale C., Jouy E., Butaye P., Franco A., Utinane A., Amado A., Moreno M., Greko C., Stärk K.D.C., Berghold C., Myllyniemi A.L., Hoszowski A., Sunde M., Aarestrup F.M. 2008. Occurrence of antimicrobial resistance among bacterial pathogens and indicator bacteria in pigs in different European countries from year 2002–2004: the ARBAO-II study. Acta veterinaria Scandinavica, 50, 1, 19–20. doi: 10.1186/1751-0147-50-19.
9. Jensen V.F., Jakobsen L., Emborg H.D., Seyfarth A.M., Hammerum A.M. 2006. Correlation between apramycin and gentamicin use in pigs and an increasing reservoir of gentamicin-resistant *Escherichia coli*. The Journal of antimicrobial chemotherapy, 58, 1, 101–107. doi: 10.1093/jac/dkl201.
10. Kehrenberg C., Mumme J., Wallmann J., Verspohl J., Tegeler R., Kühn T., Schwarz S. 2004. Monitoring of florfenicol susceptibility among bovine and porcine respiratory tract pathogens collected in Germany during the years 2002 and 2003 The Journal of antimicrobial chemotherapy, 54, 2, 572–574. doi: 10.1093/jac/dkh371.
11. Matter D., Rossano A., Limat S., Vorlet-Fawer L., Brodard I., Perreten V. 2007. Antimicrobial resistance profile of *Actinobacillus pleuropneumoniae* and *Actinobacillus porcitonsillarum*. Veterinary microbiology, 122, (1-2), 146–156. doi: 10.1016/j.vetmic.2007.01.009.
12. Morioka A., Asai T., Nitta H., Yamamoto K., Ogikubo Y., Takahashi T., Suzuki S. 2008. Recent trends in antimicrobial susceptibility and the presence of the tetracycline resistance gene in *Actinobacillus pleuropneumoniae* isolates in Japan, The Journal of veterinary medical science, 70, 11, 1261–1264. doi: 10.1292/jvms.70.1261.
13. Rayamajhi N., Sung J.S., Sang G.K., Deog Y.L., Jeong M.A., 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, 4, 359–362. doi: 10.1177/104063870501700410.

14. Sárközi R, Makrai L, Fodor L. 2018. *Actinobacillus pleuropneumoniae* serotypes in Hungary. Acta veterinaria Hungarica, 66, 343–9. doi: 10.1556/004.2018.031.
15. Sassu E.L., Bossé J.T., Tobias T.J., Gottschalk M., Langford P.R., Hennig-Pauka I. 2018. Update on *Actinobacillus pleuropneumoniae* - knowledge, gaps and challenges. Transboundary and emerging diseases, 2018, 65, 72–90. doi: 10.1111/tbed.12739.
16. 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, 4, 365–376. doi: 10.1016/s0378-1135(00)00204-2.
17. Schuwerk L., Hoeltig D., Waldmann K.H., Valentin-Weigand P., Rohde J. 2021. Sero- and apx-typing of German *Actinobacillus pleuropneumoniae* field isolates from 2010 to 2021. Veterinary research, 52, 1, 10. doi: 10.1186/s13567-020-00890-x.
18. Yoshimura, H., Takagi, M., Ishimura, M., Endoh, Y.S. 2002. Comparative in vitro activity of 16 antimicrobial agents against *Actinobacillus pleuropneumoniae*. Veterinary research communications, 26, 1, 11–19. doi: 10.1023/a:1013397419995.
19. Žutić M., Ivetić V., Radanović O., Žutić J., Jakić-Dimić D., Savić B., Pavlović I., Stanojević S. 2009. Examination of the presence of certain types of bacteria in the lungs of pigs with pneumonia. Veterinary Bulletin, 63, 1-15.

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