

ISOLATION AND IDENTIFICATION OF ACTINOBACILLUS PLEUROPNEUMONIAE IN PIG'S LUNGS AT FARMS AND THEIR SENSITIVITY TO ANTIBIOTICS

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The presence of Actinobacillus pleuropneumoniae has been established in all suspected cases of pleuropneumonia at several farms that have been included in the research. Equal incidence of pleuropneumonia has been found both among piglets for breeding and for fattening. The health monitoring of herds is extremely important, firstly because of the need for the adequate strategy to be chosen for controlling the Actinobacillus-caused pleuropneumonia and, at the same time, in order to prevent enormous economic losses that this disease may cause. The morphological, physiological and biochemical characteristics of isolated strains of Actinobacillus pleuropneumoniae were completely identical to those of referent strains. It has been found that Actinobacillus pleuropneumoniae grows most abundantly on chocolate agar when PolyVitex (bioMerieux) is used as the substrate. Out of 237 samples of altered parts of swine lungs, 13 bacterial species have been found in 193 (81%), and the incidence of Actinobacillus pleuropneumoniae within this percentage was 33%. Of all bacterial species isolated in pure culture from all investigated specimen the most dominant species were Pasteurella multocida with the incidence of 32.64% and Actinobacillus pleuropneumoniae with 29.01%. Their common incidence in all positive findings was 61.65%. If one adds to this their participation in mixed infections (2.59%), this percentage is higher (64.24%). The high percentage of these two bacterial species shows that they are at the same time the most common bacterial pathogens causing pneumonia in pigs. The incidence of other species of bacteria isolated from the lungs of diseased pigs ranged from 0.51 to 10.88%. The sensitivity of isolated strains of Actinobacillus pleuropneumoniae to the selected range of antibiotics used in clinical veterinary medicine (penicillin, ampicillin, amoxicillin, cephalosporin (III gen.) gentamycin, streptomycin, neomycin, thylosine, enrofloxacin, linkomycin-spectinomycin, tetracycline, florphenicol, trimethoprim-sulphomethoxazol and tulatromycin) was tested by the disc-diffusion method, with the implementation of antibiogram tabletes (Torlak) and

antibiogram discs (Oxoid), on chocolate agar and on Chaemophilus test medium (HTM, Biomedics). All tested strains of Actinobacillus pleuropneumoniae were sensitive to thulatromycin, while resistance of same strains was the highest to tetracycline (53%) and trimethoprim/sulphomethoxazole (56%).

Key words: Actinobacillus pleuropneumoniae, pigs, antimicrobial agents, sensitivity

INTRODUCTION

Pleuropneumonia caused by *Actinobacillus* is dominant in large agglomerations of animals and, in some countries, represents one of the biggest problems that cause a reduction of the population of pigs. These respiratory problems are frequent on our pig farms (Knežević *et al.*, 1988), where they occur as infections caused by one causative agent or, which is more often the case, the causative agent appears as an opportunist pathogen joined with other microorganisms that take part in triggering a complex of respiratory diseases. An infection may be peracute, acute or chronic, and the developed respiratory disease is very contagious and most frequently results in death. The measures taken to date in the prevention of the disease have not yielded the expected results. The most effective measure of eradication - the destruction of the threatened herd, is hardly feasible, primarily due to its high cost. Other measures for the prevention of the disease could be implemented through a programme of medicamentous prophylaxis or immunoprophylaxis, coupled with securing optimum micro-climatic and zoohygienic conditions. Application of antibiotics in prophylaxis is not possible for several reasons. Firstly, due to the trend of abolishing the use of antibiotics as means of prevention of animal diseases in many countries, and secondly, due to the possible emergence of multi-resistant strains of bacteria and the subsequent absence of any effect in therapy, as well as because of the relatively high price of antibiotics. Even with the assumption that it can be applied, medicamentous prophylaxis does not guarantee success.

The use of various types of commercial vaccines as immunoprophylactic programmes may yield satisfactory results, but it must be noted that the eradication of the disease cannot be accomplished by this type of prophylaxis. One of the ways to successfully control and eliminate the actinobacillus-caused pleuropneumonia is a timely and fast diagnostic procedure with the implementation of immunodiagnostic tests. Timely checks of the humoral immune response for *Actinobacillus pleuropneumoniae* among piglets on farms may provide for the efficient prevention of the transfer of the infecting agent within and between farms. Besides, immunological methods in diagnosing the infection caused by *Actinobacillus pleuropneumoniae* are the key for the discovery of latently infected herds, for the elimination of germ carriers from the herd, establishing the frequency of certain serotypes in the herd, control of quarantined animals and the estimation of success of the implementation of various vaccines.

The etiological agent of swine pleuropneumonia known as *Actinobacillus pleuropneumoniae* was called *Haemophilus pleuropneumoniae*, *Haemophilus parahaemolyticus*. The first data about the occurrence of this disease was given by Pattison *et al.* (1957), Matthews and Pattison (1961) and Olander (1963). An acute outbreak of a similar infection on a swine farm in Argentina was described somewhat later, in 1964, by Shope. The name *Haemophilus pleuropneumoniae* was given to the causing agent by Shope (1964) and White *et al.* (1964) and the same name was confirmed by Kilian *et al.* (1978). The name *Haemophilus parahaemolyticus* was given to strains in California by Olander (1963) and to strains from Switzerland by Nicolet *et al.* (1966, 1968). This is an immobile, gram-negative capsulated coccoidal rod, with the size of 0.4x1.0 µm. It can be observed in microscopic preparations individually or in pairs, and the optimum temperature for its growth is 37°C. It grows in anaerobic or facultatively aerobic conditions. It grows better in the presence of 5-10% of CO₂, over a period of 24-72 hours. The presence of V factor is necessary for its growth, as has been reported by Holt *et al.* (1994), Quinn *et al.* (2000), Quinn *et al.* (2002) and Washington *et al.* (2006). Unlike the majority of *Actinobacillus* species, it does not grow on MacConkey agar, but grows well on chocolate agar. The strain *Staphylococcus aureus* is used as the source of the V factor in the primoisolation of *Actinobacillus pleuropneumoniae* on blood agar. In this, colonies of *Actinobacillus pleuropneumoniae* behave as satellites, i.e. they grow along the line of staphylococci. The size of the colonies is about 1 mm, and it reduces in proportion with the distance of growth from the line of staphylococci. The colonies are partially transparent, circular, smooth, glossy, convex and surrounded by a completely transparent zone of beta-chemolysis, especially if sheep blood is used. The colonies may sometimes be waxy-gray in appearance, as reported by Quinn *et al.* (2000). For more abundant growth on chocolate or other liquid/solid agars, commercially produced NAD is added, as reported by Bilić *et al.* (1983), Žutić *et al.* (1999) and Gutierrez *et al.* (2006).

The dependence of growth of *Actinobacillus pleuropneumoniae* from the V factor is an important criterion for its identification. Besides it being a simple procedure, this is also a reason why the agar with 5-10% of sheep blood is used for primoisolation, and *Staphylococcus aureus* or *Staphylococcus epidermidis* as the source of V factor, according to Taylor (1999) and Gottschalk *et al.* (2006). Along with adding the V factor, different mediums are used in recultivation procedures, such as nutritious broth or agar, chocolate agar, MacConkey agar, PPLO agar, BHI broth and agar, as reported by many scientists: Hajsig *et al.* (1980), Bilić *et al.* (1983), Perić *et al.* (1990), Quinn *et al.* (2000) and Quinn *et al.* (2002).

Eighteen different species of *Actinobacillus* have been described since 2002 and all except three have been isolated from mammals or birds. Serologic typization *Actinobacillus pleuropneumoniae* has been performed for 15 serovarieties, with two biovarieties. Significant differences in virulence have been established among the serovarieties, as well as variations in virulence among the strains within the same serovariety. Of the virulence factors, this bacteria possesses Apx toxins, endotoxin (LPS), the capsule and the outer membrane

protein (OMP). Despite the established resistance to certain antibiotics, one may say that it is very sensitive to the majority of antibiotics, especially those in the group of penicillins and new generation antibiotics.

The diagnostics of the disease caused by *Actinobacillus pleuropneumoniae* includes a clinical examination, patho-morphological studies, bacteriological examination, serological analyses and molecular methods (PCR). The species of the genus *Actinobacillus* reduce nitrates to nitrites, are indol negative and β galactoidase and urease positive.

MATERIAL AND METHODS

The specimen used in this research were altered lungs of dead breeding and fattening piglets.

Culture media and reagents

The primary isolation of *Actinobacillus pleuropneumoniae* was performed on nutrient agar with 5-10% of sheep blood added (blood agar) in the presence of the strain *Staphylococcus aureus* or *Staphylococcus epidermidis* as the source of the V factor. Used mediums for further sub-cultivation were: chocolate agar, nutrient agar and nutrient broth (Torlak), MacConkey agar, BHI agar and BHI broth (Biomedics). Used for enriching the mediums were the vitamin supplement for bacterial growth (Hi Media) and PolyVitex (bioMerieux), according to the producers' instructions. The evaluation of dependency of growth from factor V was performed through the application of production V, X and VX discs (Hi Media). Conventional and commercial microbiological testing was performed for biochemical investigation catalase, esculin hydrolysis (bioMerieux), oxydase, urease, maltose, manythol, lactose, melobiose, arabinose, sucrose and trehalosis (Hi Media). For comparative testing the referent strain of *Actinobacillus pleuropneumoniae* ATCC 27088 was used. The following antibiotics were used for testing the sensitivity of the isolated strains of *Actinobacillus pleuropneumoniae*: penicillin, ampicillin, amoxicillin, cephalosporin (gen. III), gentamycin, streptomycin, neomycin, thylosine, enrofloxacyne, linkomycin-spectinomycin, tetracycline, florphenicol, trimethoprim-sulphomethoxazole and tulatromycin, produced by Torlak and Oxoid. In order to secure micro-aerophilic conditions (5-10% CO₂), were used Genbox CO₂ (bioMerieux).

Research methods

Specimens were inoculated on two blood agar plates each (one in aerobic conditions, another in the 5-10% CO₂ atmosphere), which were then incubated at a temperature of 37°C, during 24-72 h. The developed colonies were observed macroscopically and microscopically. Those that were morphologically characteristic, surrounded by a zone of β -haemolysis and demonstrating the satellitism phenomenon, were further sub-cultivated. For biochemical tests were used pure cultures of colonies which demonstrated the satellitism phenomenon, a well-defined hemolysis zone on agar with 5-10% of sheep blood, a positive

CAMP test with *Staphylococcus aureus*, which grew in aerobic and conditions with 10% CO₂, at a temperature of 37°C and did not grow on MacConkey agar, and which had the appearance of gram-negative coccobacilli on microscopic preparations. The antimicrobial susceptibility testing was performed on chocolate agar and on *Haemophilus* test medium (Biomedics) using the disc-diffusion method. The reading of antibacterial activity of antibiotics and the interpretational category (S, I and R) was performed on the basis of measurements of the bacteria growth inhibition zones and their comparison with the zones described in the instructions of producers of tablets/discs.

RESULTS AND DISCUSSION

Out of 237 lung samples, various species of bacteria have been isolated from 193 samples (81%), which were marked either as primary or opportunistic etiological agents of swine pneumonia. Such high percentage is the result of the fact that only pathoanatomically altered lungs were examined, but is also a confirmation of the significant role of bacteria in causing pneumonia among pigs. The number of samples from farms that were included in the research varied, i.e. it depended on the number of animals on a farm and on the incidence of respiratory infections. Out of 237 tested lung samples originating from pigs, 13 species have been isolated from 193 (81%) and, within the said percentage, the incidence of *Actinobacillus pleuropneumoniae* was 33% (Figure 1).

Two species of bacteria dominated the spectre of microorganisms found in the lungs: *Pasteurella multocida* with the incidence of 32.64% and *Actinobacillus pleuropneumoniae* with 29.01%. Their incidence in all positive findings was 61.65%. If one adds to this their participation in mixed infections (2.59%), this percentage is higher - 64.24%. The high percentage of these two bacterial species shows that they are at the same time the most common bacterial pathogens causing pneumonia among pigs. Namely, lung samples from dead pigs were taken at times when pneumonias appeared, with the purpose of making the etiological diagnosis, finding the most efficient antibiotic *in vitro* and its implementation in pneumonia therapy in the herd. During the primary isolation of *Actinobacillus pleuropneumoniae* from lungs, the strain *Staphylococcus aureus* was inoculated on blood agar as the source of the V factor. In growth, colonies of *Actinobacillus pleuropneumoniae* demonstrated the phenomenon of satellitism, i.e. they grew more abundantly along the staphylococci line (Figure 2).

The isolated strains of *Actinobacillus pleuropneumoniae* were sensitive to the majority of antibiotics used. All examined strains were sensitive to thulatomycin, while the percentage of resistance to tetracycline was 53% and 56% to trimethoprim/sulphomethoxazole, respectively. Similar resistance percentages of the strains of *Actinobacillus pleuropneumoniae* to trimethoprim/sulphomethoxazole (54% of cases), to tetracycline (70%) and to apramycin (84%) were found by Bilić (1992).

The sensitivity of the strains of *Actinobacillus pleuropneumoniae* to penicillin, ampicillin and linkomycin-spectinomycin has been observed for years, according to data presented by Gilbride and Rosendal (1984), Suzuki *et al.*

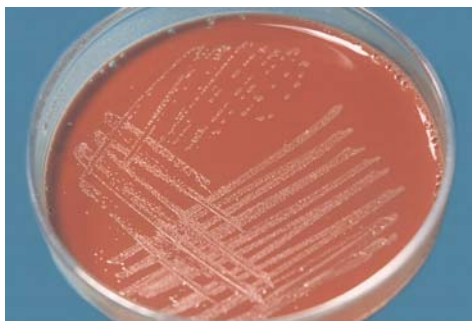


Figure 1. Appearance of *Actinobacillus pleuropneumoniae* colonies on chocolate agar

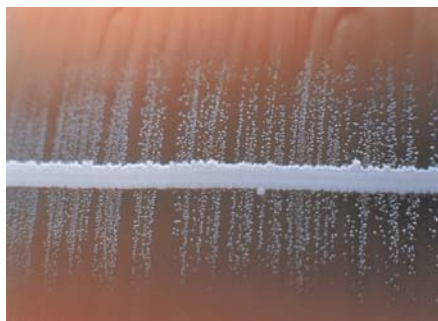


Figure 2. Appearance of *Actinobacillus pleuropneumoniae* colonies and the phenomenon of *Staphylococcus aureus* satellitism

(1989), Lipej *et al.* (1990), Bilić (1992), Žutić *et al.* (1999), Yoshimura *et al.* (2002), which corresponds to the results obtained in this research. Also, Gottschalk and Taylor (2006) established the sensitivity of *Actinobacillus pleuropneumoniae* to fluoroquinolones (enrofloxacin) and semi-synthetic cephalosporins (ceftiofur). In the *in vitro* examination of sensitivity of 121 strains of *Actinobacillus pleuropneumoniae* to 14 anti-microbial agents, authors have established high sensitivity to penicillin, ampicillin, amoxicillin, neomycin, chloramphenicol, linkomycin-spectinomycin and furazolidone.

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IZOLACIJA I IDENTIFIKACIJA *ACTINOBACILLUS PLEUROPNEUMONIAE* IZ PLUĆA SVINJA U FARMSKIM USLOVIMA DRŽANJA I NJIHOVA OSETLJIVOST NA ANTIBIOTIKE

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SADRŽAJ

U novije vreme, pleuropneumonija izazvana aktinobacilusom dominira u velikim aglomeracijama životinja i u nekim zemljama predstavlja jedan od problema koji značajno doprinosi smanjenju populacije svinja. Navedeni problem, postoji i na našim farmama svinja gde protiče kao infekcija izazvana jednim uzročnikom, ili se, što je češća pojava, uzročnik javlja kao oportunistična patogena bakterija udružena sa drugim mikroorganizmima koji učestvuju u nastanku kompleksa respiratornih bolesti. Iz navedenih razloga monitoring zdravstvenog stanja jedinki na farmi je neophodan ne samo zbog utvrđivanja odgovarajuće strategije za kontrolu pleuropneumonije koju izaziva aktinobacillus, već i radi sprečavanja ogromnih ekonomskih gubitaka koje ova bolest može da prouzrokuje. U svim slučajevima sumnje na pleuropneumoniju kod svinja sa nekoliko farmi koje su bile obuhvaćene ispitivanjem, ustanovljeno je prisustvo *Actinobacillus pleuropneumoniae*. Podjednaka zastupljenost pleuropneumonije ustanovljena je, kako kod prasadi u odgoju, tako i kod prasadi u tovu. Od 237 uzoraka promenjenih delova pluća uginulih svinja iz 193 (81%) izolovano je 13 vrsta bakterija, a u okviru navedenog procenta zastupljenost *Actinobacillus pleuropneumoniae* je iznosila 33,15%. Morfološke, fiziološke i biohemijske osobine izolovanih sojeva *Actinobacillus pleuropneumoniae* potpuno su bile identične sa osobinama referentnog soja. Utvrđeno je da *Actinobacillus pleuropneumoniae* najobilnije raste na čokoladnom agaru kada se kao supstrat za obogaćenje koristi PolyVitex (bioMerieux). Od bakterija koje su iz pluća izolovane u čistoj kulturi najzastupljenije su bile *Pasteurella multocida* sa 32,64% i *Actinobacillus pleuropneumoniae* sa 29,01%. Njihovo zajedničko učešće u ukupno pozitivnim nalazima iznosi 61,65%, a ako se tome doda i njihovo učešće u mešanim infekcijama od 2,59 % onda taj procenat

iznosi 64,24%. Ovako visoki procenti zastupljenosti obe vrste bakterija ukazuju na njihovu značajnu ulogu u nastanku pneumonije kod svinja. Zastupljenost ostalih vrsta bakterija izolovanih iz pluća obolelih svinja kretala se od 0,51% do 10,88%. Kod izolovanih sojeva *Actinobacillus pleuropneumoniae* osetljivost na odabrani broj antibiotika koji se koriste u kliničkoj praksi (penicilin, ampicilin, amoksicilin, cefalosporine (III gen.), gentamicin, streptomycin, neomicin, tilozin, enrofloxacin, linkomicin-spektinomycin, tetraciklin, florfenikol, trimetoprim-sulfometoksazol i tulatromicin) ispitivana je disk-difuzionom metodom, primenom antibiogram tableta (Torlak) i antibiogram diskova (Oxoid), na čokoladnom agaru i na hemofilus test medijumu (HTM, Biomedics). Svi ispitivani sojevi *Actinobacillus pleuropneumoniae* bili su osetljivi na tulatromicin, a najveća rezistencija kod istih sojeva, ustanovljena je na trimetoprim/sulfometoksazol od 56% i na tetraciklin od 53%.