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Original paper

Antimicrobial susceptibility of pathogenic bacteria isolated from swine lungs

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Abstract

The aim of this study was to determine the antibiotic susceptibility of bacterial strains isolated from pathological lungs of pigs with respiratory symptoms in a farm from Western Romania. From pigs that died with respiratory signs there were isolated eight *Actinobacillus pleuropneumoniae*, six *Streptococcus suis*, four *Escherichia coli* and two *Salmonella choleraesuis* strains.

Antibiotic susceptibility has tested for oxytetracycline, penicillin, enrofloxacin and florfenicol. The results of the study demonstrated that more than 80% of *A. pleuropneumoniae* strains were sensitive to florfenicol. Over 95% of the Gram-negative isolates were resistant to penicillin and oxytetracycline. However, observations from last years indicate a decreased sensitivity of *A. pleuropneumoniae* strains to penicillin and oxytetracycline. An increasing level of resistance to antimicrobials used frequently has been also observed among *Salmonella* strains. Although other studies have indicated that these strains have preserved total sensitivity to gentamicin, in our study, 50% of *Salmonella* strains were only intermediate to this antibiotic. However, gentamicin and florfenicol seem to remain the antimicrobials with best efficiency for all Gram-positive and Gram-negative bacterial strains isolated in this study.

Keywords Antimicrobial susceptibility,respiratory diseases, bacteria, swine

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Introduction

Porcine respiratory disease complex (PRDC) is a syndrome caused by mixed viral (porcine respiratory and reproductive syndrome virus, porcine circovirus type 2, porcine coronavirus) and bacterial pathogens (*Actinobacillus pleuropneumoniae, Pasteurella multocida, Mycoplasma hyopneumoniae, Streptococcus suis, Eschericha coli*) (M. HAIMI-HAKALA & al. [1]). In many cases it is difficult to say what is the primary pathogen and which one acts as a predisposing agent for other infections or as a secondary infection (E. THACKER & al. [2]; M.S. HANSEN & al. [3]; V. FACHINGERA& al. [4]). Many of these pathogens can also be found in clinically healthy pigs (A. PALZER & al. [5]). Smilar studies in this field have been published by S. BARAITAREANU & al. [6]; V. HERMAN & al. [7]; and C. PASCU & al. [8].

Pleuropneumonia is one of the most important respiratory diseases in pigs. *A. pleuropneumoniae* is present in the nasal cavities and tonsils of healthy pigs, but is able to colonize the lungs causing high morbidity and mortality due to acute fibrinous pleuropneumonia. Animals that survive the initial infection frequently have chronic lung lesions.

Pasteurella multocida in the most common bacterial species among swine respiratory disease (SRD) being involved in evolution of atrophic rhinitis which typically affected young pigs.

S. suis is a third major respiratory pathogen in pigs, but in addition to being responsible for pneumonia, *S. suis* can cause a variety of diseases including meningitis, arthritis, peri- and endocarditis, polyserositis, septicaemia, abortion and sudden death.

High stocking density in a closed environment facilitates transmission of airborne pathogens within the pigs and between farms as well. Increased use of antimicrobials, broadly administered both for therapy and prophylaxis is closely associated with frequent occurrence of the disease. As any exposure of bacteria to antibiotics may lead to selection of resistance, antimicrobial susceptibility should be carefully monitored. Knowledge about trends over time is important to ensure long-term efficacy of the antimicrobials.

The purpose of this study was to determine the bacterial aetiology of respiratory disorders and to determine minimum inhibitory concentration (MIC) of 5 antimicrobial agents currently applied in swine farms against respiratory pathogens.

Materials and Methods

1. Sampling procedures

A total of 20 isolates from pneumonic lungs were obtained in the course of the routine diagnose. Samples were only taken from diseased or recently deceased animals with acute clinical signs of respiratory tract infections, not exposed to antibacterial treatment for at least 15 days prior to sampling. These pigs came from farms in the west of Romania with a history of respiratory symptoms.

The samples were plated on nutrient agar and nutrient broth (Oxoid), nutrient agar with 5% fresh defibrinated sheep blood, and MacConkey agar (Oxoid). All plates were incubated at 37ºC in air condition for 24 h. After primary isolation, the isolates were purified and subcultured on special media, or on media with supplements.

The isolates were purified and characterized by morphological criteria and using standard biochemical tests, commercially available identification system (such as API Microbial Identification Kits, bioMerieux).

The primary isolation of *Actinobacillus pleuropneumoniae* was performed on blood agar in the presence of the *Staphylococcus aureus* as the source of the V factor (**Figure 1**).

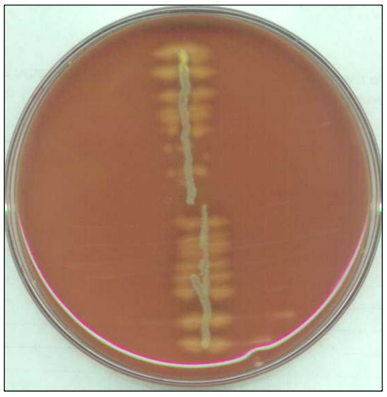

Figure 1. Positive CAMP test for an *A. pleuropneumoniae* strain with *S. aureus*

The media for sub-cultivation were: chocolate agar, MacConkey agar, BHI agar and broth (Oxoid). The evaluation of dependency of growth from factor V was performed through the application of production V, X and VX discs (HiMedia).

Commercial microbiological testing was performed for biochemical investigation catalase, esculin hydrolysis

(API ZYM, bioMerieux), oxydase, urease, maltose, manythol, lactose, melobiose, arabinose, sucrose and trechalosis (HiMedia).

The developed colonies were observed macroscopically and microscopically. Those that were morphologically characteristically, 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% of sheep blood, a positive CAMP test with *S. aureus*, which grew in aerobic conditions, at a temperature of 37ºC and did not grow on MacConkey agar, and which had the appearance of gram-negative coccobacilli on microscopic preparations.

The primary isolation of *Streptococcus suis* was performed on blood agar and incubated at 37ºC for 24-48 h. All small colonies, surrounded by a zone of β-haemolysis, and which had the appearance of gram-positive cocci, arranged in chains of different sizes was taken in consideration (**Figure 2**, **Figure 3**). Biochemical identification and determined of the species was performed with API 20 Strep (bioMerieux).

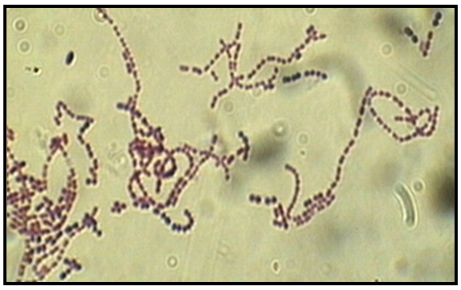

Figure 2. *Streptococcussuis* broth culture, Gram staining.

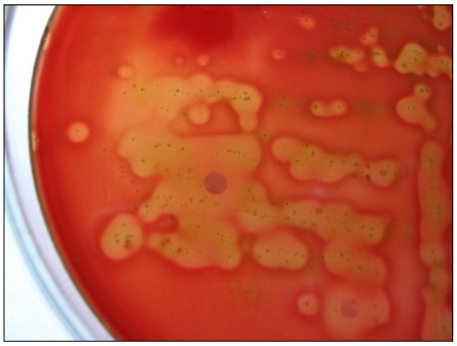

Figure 3. *Streptococcus suis* – hemolysis on blood agar.

The primary isolation of the *Enterobacteriaceae* has performed on usual media (nutritive broth and agar, Oxoid). The colonies were subcultured on selective media – TSI, MIU, Levine and MacConkey (Oxoid).

E. coli strains were identified based on morphology - gram-negative coccobacilli on microscopic preparations, green colonies on Levine and biochemical activity on API 20E (bioMerieux) (**Figure 4**).

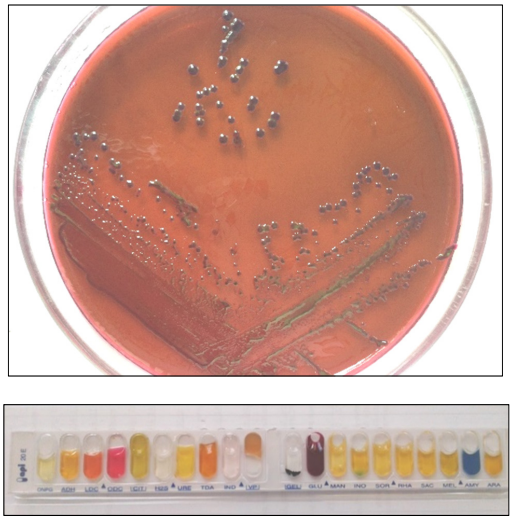

Figure 4. *E. coli* strain on Levine agar (up) and its biochemical activity on API 20E (down).

For *Salmonella* strains, identification has performed with evidence of hydrogen sulphide production, indole negative, motility and fermentative activity. Species determination was made using API 20E (bioMerieux).

The bacterial strains that are identified were *A. pleuropneumoniae* (8 strains), *S. suis* (6 strains), *E. coli* (4 strains) and *S. choleraesuis* (2 strains).

2.2. Antimicrobial susceptibility testing

A broth microdilution was used to determine MIC and breakpoint values. The MIC was considered to correspond to the first dilution at which no bacterial growth was detected. The antimicrobials tested were chosen from antimicrobials frequently used in pig farms and in respiratory disorders.

The dilution range for determination of MICs for florfenicol was 0.03 to 64 µg/ml, for enrofloxacin was 0.03 to 250 µg/ml, for gentamicin was 0.5 to 250 µg/ml, for

penicillin was 1 to 28 µg/ml, and for oxytetracycline was 0.01 to 64 µg/ml.

There are two methods to obtain MIC values: broth dilution and agar dilution (CLCSI [6]). In this study it was used the broth dilution method designed by us, using micro-well plates (microdilution method).

Pure cultures were subcultures on Columbia blood agar (10%) and Columbia agar (Oxoid) and incubated for 24 hours at 37°C. From each plate, there were taken colonies and dissolved in 4 ml saline until the turbidity was 0.5 on McFarland scale. This turbidity corresponds to a bacterial concentration 1-2 x 10^8 CFU / ml.

From this suspension, 50 µl were transferred in 10 ml nutritive broth and in 10 ml nutritive broth with lacked horse blood (Oxoid) 2% for fastidious bacteria. The final concentration of inoculum was 1-5 x 10^5 CFU/ml. This inoculum had to be used in 15 minutes after it was obtained (CLCSI [9]).

As antimicrobials, there were used injectable formulations, with the mentioned concentrations, as it follows florfenicol (300 mg / ml), enrofloxacin (5%), gentamicin (5%), penicillin (400.000 IU / fl), and oxytetracycline (200 mg/ml). Each antimicrobial was diluted first up to a concentration of 1000 µg / ml. From this solution, it has made a serial dilution in Mueller-Hinton broth, in microwell plates up to a final concentration of 0.005 µg / ml. For each bacterial strain, there were used two rows (24 microwells).

The layout of the test components in the well plate was as follows:

- In microwells A1, C1, E1, G1 – 200 µl antimicrobial solution (1000 µl / ml);
- Starting with microwells A2, C2, E2 and G2 – 100 µl antimicrobials diluted according to the methodology described above + 50 µl bacterial inoculum**.**

On each microwells plate there was tested one bacterial strain and four antimicrobials.

Microtiter plates were incubated at 35°C ± 1°C and the results were read visually by the same person after 20 hours and, for comparison reasons, after 24 hours of incubation (**Figure 5**).

MIC values corespond to the last antimicrobial dilution which visibly inhibited the bacterial strain growth (may appear either as turbidity or in the form of a deposit on the bottom of the well).

Interpretation of the results was made according to the CLCSI guidelines [10].

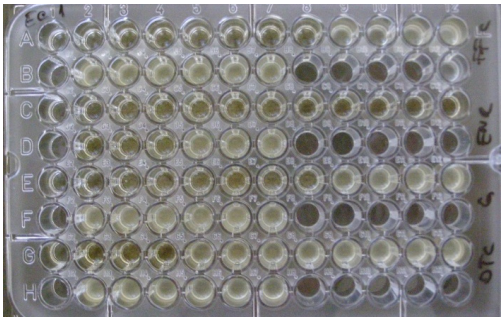

Figure 5. Microtiter plate aspect after incubation period containing a *E. coli* strain.

Results and Discussions

MIC was reported as susceptible, intermediate and resistant, where CLSI veterinary breakpoints were available (CLSI, 2013 [10]).

Table 1 summarizes the susceptibilities of the isolates to 5 antimicrobials.

Table 1. MIC of five antimicrobials against bacteria isolated from swine lungs.

Antimicrobial	**MIC (µg/mL)**		
	Min	**Max**	**Mode**
Florfenicol	0.12	8	4
Enrofloxacin	0.01	32	0.5
Gentamicin	4	8	4
Penicillin	0.06	0.12	0.12
Oxytetracycline	0.12	2	1

The results obtained show that for the tested gram negative bacteria the most efficient antimicrobials were florfenicol and gentamicin and for gram-positive bacteria (streptococcal strains) was only penicillin.

The resistance phenomenon was registered for oxytetracycline in all tested strains.

From gram-negative bacteria, *A. pleuropneumoniae* was sensitive only to florfenicol, *E. coli* strains were sensitive to florfenicol and gentamicin, and *Salmonella* strains were sensitive to enrofloxacin and florfenicol.

H.J. WISSELINK & al. [11] in Denmark and Sweden, who observed streptococcal strains resistance tendency to more than one antimicrobial, including tetracycline resistance, obtained similar results.

Retrospective studies have been published that investigated the antimicrobial susceptibility of *A. pleuropneumoniae* isolates from swine. M. ARCHAMBAULT & al. [12] reported the antimicrobial susceptibilities of 43 isolates of *A. pleuropneumoniae* from Canada in which all isolates were 100% susceptible to ceftiofur, florfenicol, enrofloxacin, erythromycin, clindamycin, TMP-SMX, and tilmicosin, but reported a low level of susceptibility to chlortetracycline and oxytetracycline (11.6% and 9.3% susceptibility, respectively). M. VANNI &al. [13] also showed low rates of susceptibility for tetracycline (< 17%) and penicillin (< 15%). F. El GARCH & al. [14] reported the susceptibilities of 158 *A pleuropneumoniae* isolates that showed 100% susceptibility to ceftiofur, amoxicillin-clavulanate, and tulathromycin with 96% to 99% susceptibility to florfenicol, tilmicosin, and enrofloxacin, while tetracycline susceptibility was reported at 70%.

In Spain, C. GUTIERREZ-MARTIN & al. [15] observed a considerable increase of *A. pleuropneumoniae* strains tetracycline resistance, comparing to *A. pleuropneumoniae* strains isolated a few years ago.

In other studies (M. HAIMI-HAKALA & al. [1]; Z. KUCEROVA [16])*A. pleuropneumoniae* strains were resistant to tetracycline and penicillin, and sensitive to florfenicol. Streptococcal strains were sensitive to penicillin (A. de JONG & al. [17]; C. FABLET & al. [18]).

D.A. DAYAO & al. [19] in a study to determine the antimicrobial resistance profile observed the same resistance to tetracycline in Australia. The majority of *A. pleuropneumoniae* isolates were resistant to erythromycin (89%) and tetracycline (75%). This resistance is the consequence of tetracycline administration in food, both as growing promoter and as prophylactic measure.

Our findings suggest that *A. pleuropneumoniae* is widely prevalent in pig farms; we conformed that infections caused by *A. pleuropneumoniae* are more common than those caused by *Streptococcus suis*, *E. coli* and *Salmonella choleraesuis* ($P < 0.01$).

Even if the percent of sensitive strains to gentamicin was quite high, the fact that gentamicin is absorbed less administered orally, the best absorption was obtained by administering i.m., makes this antimicrobial not very useful in widespread practice in the treatment of swine respiratory infections.

These analyses display several significant differences between antimicrobials, such as oxytetracycline, significant differences ($P < 0.01$) were noted between isolates from the last 5 years and isolates from the last 10 years.

The present of multi-resistance bacterial strains and the resistance to penicillin, oxyteracycline, enrofloxacin may be the consequence of excessive administration of antibiotics in pig farm. There is a continuous debate in veterinary medicine regarding the use of antimicrobials in food animals. This potential threat to human health resulting from improper antimicrobials use in animals is significant, as pathogenic-resistant bacteria could be widely be disseminated through the food chain, but also into the water bodies (SALA & al. [20]; MARINESCU & al. [21]).

Conclusions

The results of this study were consistent with those from other studies. Most isolates were resistant to oxytetracycline. This resistance is a consequence of administration in food, such a growth promoter or prophylactic. The data presented in this paper indicate the importance of prudent use of the antimicrobials when treating respiratory diseases or other infections in pigs.

For most antibiotics and pathogens the percentage resistance increased compared with previous data. The surveillance of *in vitro* susceptibility of these pathogens must continue to avoid the emergence and dissemination of antimicrobial resistance.

Monitoring antimicrobial susceptibility provides valuable information about changes that may occur in the antimicrobial sensitivity of these bacteria and, therefore, it is an important tool in therapy.

Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article. The manuscript was read and approved by allauthors.

Compliance with ethical standards

Any aspect of the work covered in this manuscript has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

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