Diagnostics of main bacterial agents of porcine respiratory diseases complex (PRDC) using PCR detection of *Mycoplasma hyopneumoniae*

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ABSTRACT: The main goal of our work is the presentation and analysis of incidence of porcine respiratory disease complex (PRDC) regarding bacterial agents in the territory of northern districts of Slovakia. *Mycoplasma hyopneumoniae* and other secondary bacterial causative pathogens of PRDC comprised 75.2% of all cases (98) with clinical signs of respiratory infections that we examined in the course of one year. We present also one of possibilities to the solution of problematic detection of *M. hyopneumoniae* which is, like the whole rank of mycoplasmas, very difficult to cultivate. This problem was solved by using the PCR method with the direct isolation of *M. hyopneumoniae* from lungs tissue. In antibiotic sensitivity testing of *Pasteurella multocida* and *Actinobacillus pleuropneumoniae* resulted enrofloxacin as the most effective antibiotics in the therapy of PRDC regarding bacterial agents.in above mentioned territory.

Keywords: pigs; *Pasteurella multocida*; *Actinobacillus pleuropneumoniae*; *Mycoplasma hyopneumoniae*; antibiotic sensitivity; PCR

Porcine respiratory disease complex (PRDC) is an economically significant respiratory disorder characterized by slow growth, decreased feed efficiency, lethargy, anorexia, fever, cough, and dyspnea. Diagnostic laboratories have isolated multiple pathogens from cases of PRDC, including porcine reproductive and respiratory syndrome virus (PRRSV), *Mycoplasma hyopneumonia*e, swine influenza virus (SIV), *Actinobacillus pleuropneumoniae*, and pseudorabies virus (PRV) (Van Reeth *et al*., 1996). Of these pathogens, PRRSV, *M. hyopneumoniae*, and SIV are most frequently detected in 10- to 22-week-old pigs with clinical signs of PRDC (Thacker *et al*., 2001). *M. hyopneumoniae* is recognized as the causative agent of porcine enzootic pneumonia (EP), a mild, chronic pneumonia commonly complicated by opportunistic infections with other bacteria (Ross, 1999). The primary clinical sign associated with *M. hyopneumoniae* infection is a sporadic, dry, nonproductive cough. Other clinical signs, such as fever or impaired growth, are linked to secondary invaders, especially *Pasteurella multocid*a (Thacker *et al*., 1999). Typical mycoplasmal pneumonia lesions consist of well-demarcated dark-red-to-purple (acute) or tan-grey (chronic) areas of cranioventral consolidation. Microscopic examination reveals bronchopneumonia with suppurative and histiocytic alveolitis with peribronchiolar and perivascular lymphohistiocytic cuffing and nodule formation, typical of hyperplasia of bronchoalveolar lymphoid tissue. The disease has a worldwide distribution and causes considerable economic losses in swine production due to reduced growth rate and feed conversion efficiency (Baumeister *et al*., 1998). The detection of *M. hyopneumoniae* is usually based on the isolation of the organisms by culture or by immunofluorescence tests with lung sections (Armstrong *et al*., 1984). The cultivation of *M. hyopneumoniae* is difficult due to the fastidious culture requirements and the extremely slow growth of *M. hyopneumoniae*, often resulting in overgrowth by other mycoplasmas colonizing the respiratory tracts of pigs (Friis, 1975). Cross-reactions with *Mycoplasma flocculare* and *Mycoplasma hyorhinis* re-

duce the specificity of conventional immunological detection methods (Bolske *et al*., 1987).

P. multocida in pigs is very important pathogen, namely as a secondary infectious agents. *P. multocida* is widespread in breedings and a great number of animals are their carriers. Multiplication of this pathogen occurs in the case of diminished natural resistance that happens on consequence of environmental factors or other infectious and non-infectious diseases. Infection is transmitted mostly aerogenously (Kamp *et al*., 1996). *P. multocida* multiplicate quickly in the place of their invasion and in the short period of time they deluge various organs and tissues. Endotoxins, they liberate from microbes, do the damage mainly to the lung tissue and cause necrotic changes. Lesions are confined to the thoracic cavity and are superimposed on those of *M. hyopneumoniae*. Typically, anteroventral consolidation of the lung is seen, together froth in the trachea (Pijoan and Fuentes, 1987). The Tox-A protein is an essential virulence factor for progressive atrophic rhinitis. Toxigenic strains of *P. multocida* were first reported by Pijoan *et al*. (1984). The role, if any, of toxigenity in pneumonic pasteurellosis is still under debate. For example, Hoie *et al*. (1991) found that 94% of serotype A and 90% of serotype B isolates from pneumonic lungs were toxigenic. In contrast, Rubies *et al*. (1996) found no toxigenic strains (either A or D) in 218 isolates from pneumonic lungs in Spain. Nevertheless, the detection of Tox-A is a very frequently used method for determination of pathogenicity of *P. multocida* isolates at present (Lichtensteiger *et al*., 1996; Satran *et al*., 1999).

Actinobacillus pleuropneumoniae is the etiological agent of swine pleuropneumonia, a respiratory disease that continues to have a worldwide economic impact. *A. pleuropneumoniae* induces a highly contagious disease characterised by acute or chronic fibrinohaemorrhagic necrotising pneumonia, in which moist, yellowish pleural adhesions with massive fibrin infiltration are more common (Sebunya and Saunders, 1983). Transmission of the disease appears to occur directly from an infected pig to a susceptible pig, since *A. pleuropneumoniae* is not known to survive long in the surrounding enviroment (Willson *et al*., 1987). Two biotypes of *A. pleuropneumoniae* are recognised. Biotype 1 is NAD dependent and biotype 2 is NAD independent. So far, 14 serotypes have been recognised, 12 within biotype 1 and 2 within biotype 2 (Nicolet, 1988). Serotypes are generally distributed by geographic location.

In this work, besides the summary of our examination results, we present the survey of incidence of *M. hyopneumoniae*, *P. multocida* and *A. pleuropneumoniae* mutual connection and their chronological occurrence in the period of one calendar year in north areas of Slovakia (Figure 1).

MATERIAL AND METHODS

Reference bacterial strains and samples

Ninety eight cadavers of perished animals with signs of respiratory disorders were investigated. 5 to 10 grams of tissue was taken from changed parts of lungs.

Collection strains *Pasteurella multocida* CAPM 6077 T, *Actinobacillus pleuropneumoniae* CAPM 3888 *and Bordetella bronchyseptica* CAPM 5956 were used as a reference material for the cultivation microbiological diagnostics. The reference strain HL-47 (MEVAK,

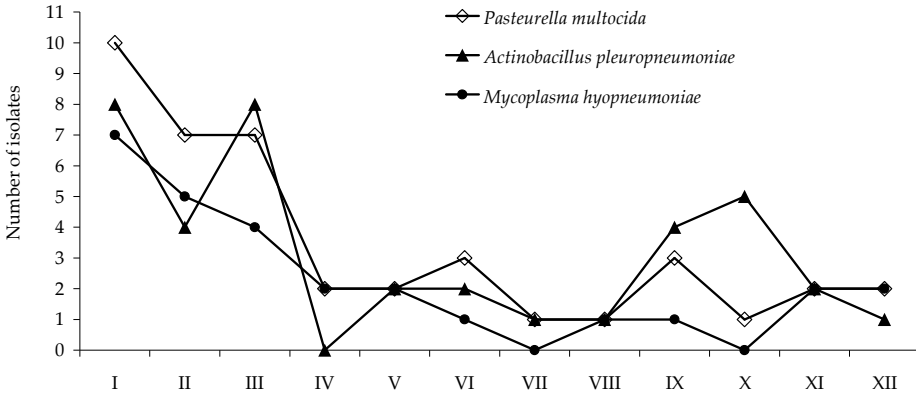

Figure 1. Chronologic incidence of *Mycoplasma hyopneumoniae*, *Actinobacillus pleuropneumoniae*, and *Pasteurella multocida* in north area of Slovakia in course of the year 2001

Slovakia) was used as the positive control for the PCR detection of *Mycoplasma hyopneumoniae.*

Cultivation

To proof *Pasteurella* and *Actinobacillus* species the cultivation method on the blood agar, Gram's stain, catalasic test and the tube test for the carbohydrate fermentation were used by Bergey's manual (Holt *et al*., 1994). Important biochemical characteristics of *P. multocida* included positive reactions for catalase, indole and oxidase.

Colonies of *A. pleuropneumoniae* produced increased zone of hemolysis within the zone of partial lysis surrounding a beta-toxinogenic *Staphylococcus aureus* (CAMP phenomenon). Important biochemical characteristics were positive reactions for urease, mannitol, xylose and ribose.

For the PCR detection of *M. hyopneumoniae* we used the modification of the method according to Mattsson *et al*. (1995) and Harasawa *et al*. (1991). The modification was based on direct DNA extraction from lung tissue described bellow.

DNA extraction from lung tissue

One to two grams piece (0.5 × 0.5 × 0.5 cm) of lung tissue (fresh or frozen) we cut and minced in a petri dish with the aid or two scalpels. We cut the tissue carefully into pieces as small as possible, then we added 500 µl of phosphate buffered saline (PBS) and continued cutting.

We transfered 300 µl of the suspension (without any pieces of tissue) to a microcentrifuge tube and added 385 µl of STE buffer (100 mM NaCl, 50 mM Tris-HCl buffer pH 7.4, and 1 mM EDTA), 5 µl of proteinase K solution (20 mg/ml) and 10 µl of 20% SDS – sodium dodecil sulphate. All buffers and substances of solutions are described in Kauffman *et al*. (1995).

The samples were mixed by vortex and incubated for 4 h at 50°C.

The phenol extraction and DNA precipitation with ethanol were used by Kauffman *et al*. (1995).

In vitro amplification by PCR

One milliliter of broth culture of each bacterium *Mycoplasma hyopneumoniae, Pasteurella multocida, Bordetella bronchyseptica, Actinobacillus pleuropneumoniae* was centrifuged, washed in phosphate-buffered saline (PBS), resuspended in water, and lysed by heating at 100°C for 5 minutes. DNA from 5 µl samples of undiluted and 10-fold-diluted lysed cells was used as DNA templates for specificity determination of PCR amplification.

A 649-bp fragment of the *16S rRNA* gene from *M. hyopneumoniae* was amplified by PCR with the forward primer 5′- GAG CCT TCA AGC TTC ACC AAG A- 3′ (nucleotide positions 212 to 233 in the *16S rRNA* sequence) and the reverse primer 5′- TGT GTT AGT GAC TTT TGC CAC C - 3′ (nucleotide positions 839 to 860). The amplification was performed in a 50 µl reaction mixture in accordance with protocol by Mattsson *et al*. (1995).

Antibiotic-sensitivity testing

The resistance was tested by the disc diffusion method in petri dishes containing in *Actinobacillus pleuropneumoniae* Haemophilus test medium base (HTM) with HTM supplement (OXOID) enriched with Virox (OXOID) as recommended by the manufacturer. In *Pasteurella multocida* it was on the Mueller-Hinton agar (OXOID). Antibiotic sensitivity was defined using Antimicrobial susceptibility test discs (OXOID). The strains to be tested were first incubated at 37°C overnight and then the cultures were resuspended in PBS. The density of the suspension was adjusted to 0.5 MacFarland (1 to 4×10^8 CFU/ml). The plates were inoculated by spilling the suspension over the surface. Inhibition zones of the individual drugs were read after 24 h of incubation at 37°C and strains were classified as sensitive, intermediary and resistant according to international standards (NCCLS, 1997).

RESULTS AND DISCUSSION

In the year 2001 we examined altogether 98 cases of perished animals – pigs with the respiratory apparatus distortions. *Pasteurella multocida* was isolated from 41 cases (44%), *Actinobacillus pleuropneumoniae* from 38 cases (40.8%) and *Mycoplasma hyopneumoniae* from 27 cases (29%). The most frequent separate isolate was *P. multocida* (14 cases), the less frequent one was *M. hyopneumoniae* (4 cases). In five cases we noted a common occurrence of all three pathogenic agents. The most frequent combi-

nation was the occurrence of *M. hyopneumoniae* and *P. multocida* (11 cases).

The number of the respiratory infection cases in pigs culminated in the months January–March, whereby in the summer we noted only one case in a month (Figure 1).

Numbers given above result in a fact that *P. multocida* is the most serious bacterial pathogen of the pig's respiratory tract in the investigated area. *P. multocida* co-operates together with *M. hyopneumoniae* in the porcine enzootic pneumonia (EP). In our statistics this situation happened for 11 times and for 5 times it functioned together with *A. pleuropneumoniae*. The role of *A. pleuropneumoniae* in this epizootological study is in principle reduced in the aspect of a relatively regular outbreak recurrences of the infectious pleuropneumonia (AAP) in two pig farms of one district. Here unsuitable zoohygienic breeding conditions, as well as insufficient decontamination measures when overcoming the AAP – infection are considered to be a very important factor.

It is worth to notice the survey on the occurrence of *M. hyopneumoniae*, which is possible to be effectively diagnosed using the PCR method. The specificity of amplification is shown on the Figure 2. In comparison with past years, the diagnostic record of this pathogen in our workplace has increased up to 60 to 70% from the amount of indicated cases. In comparison with latest years, the number of EP cases raised 2 to 3 times.

The biggest problem from the point of view of prevention and therapy of the EP – AAP syndrome represents a relatively variable species virulence and a considerable ATB – resistance spectrum of *A. pleuropneumoniae*.

A. pleuropneumoniae is particularly susceptible *in vitro* to penicillin, ampicillin, cephalosporin, chloramphenicol, tetracyclines, colistin, sulfonamide, and gentamycin, to which it has low minimum inhibitory concentration (MIC) (Prescott and Baggot, 1993). High MIC values are found for streptomycin, kanamycin, spectinomycin, spiramycin and lincomycin (Nicolet and Schifferli, 1982; Gilbride and Rosendal, 1983; Inoue *et al*., 1984; Nadeau *et al*., 1988).

We tested isolated strains for their antibiotic sensitivity. The results on the Figure 3 represent total 78 strains of *A. pleuropneumoniae* isolated during two years (2000–2001). The highest level of resistance of the tested strains during two years was the resistance to streptomycin (90%), the highest sensitivity of isolates was to enrofloxacin (100%). Our results generally correspond with result of ATB testing in strains isolated in the Czech Republic, where the strains selected during 1999–2000 most frequently demonstrated resistance to streptomycin (70%) and erythromycin (95%), and no strain was resistant to norfloxacin (Satran and Nedbalcova, 2002).

In *P. multocida*, we generally detected a wide sensitivity spectrum with exception of tylosine (Figure 4). The frequency of obtained resistance of *P. multocida* to tylosine is usually higher and *A. pleuropneumoniae* is completely resistant to this kind of antibiotic. The results in 90 isolates of *P. mulrocida* were obtained according the same time as above mentioned *A. pleuropneumoniae* ATB sensitivity testing results.

From the results of the sensitivity testing, enrofloxacin seems to be the most effective antibiotic referring to all mentioned pathogens. It has also a good effectiveness on *M. hyopneumoniae*, the resist-

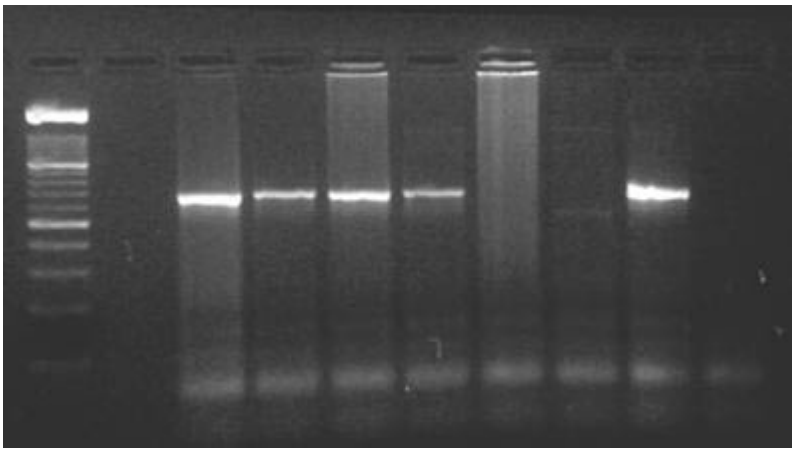

Figure 2. PCR detection of *Mycoplasma hyopneumoniae*

Lanes: 1 – 100 bp MW DNA standard, 2 – empty, 3–6 – samples (lung tissue), 7 – *Bordetella bronchyseptica*, 8 – *Pasteurella multocida*, 9 – *Mycoplasma hyopneumoniae* (649 bp), 10 – negative control

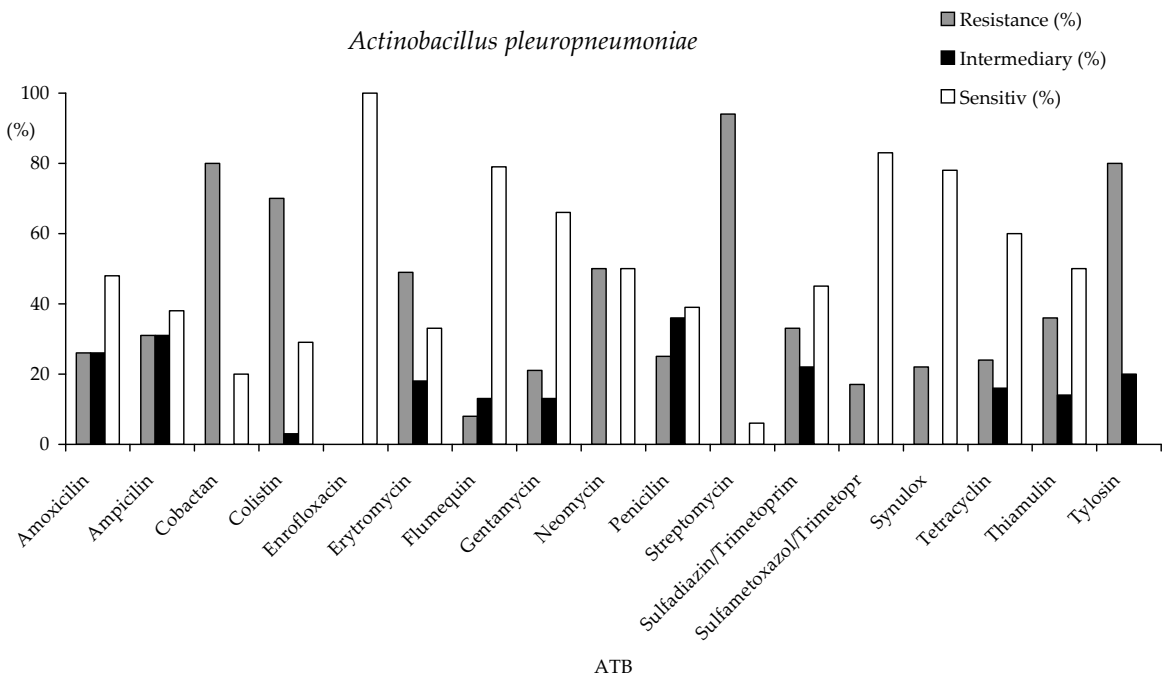

Figure 3. The results of antibiotic resistance testing of *Actinobacillus pleuropneumoniae* strains isolated in course of years 2000–2001 (78 isolates)

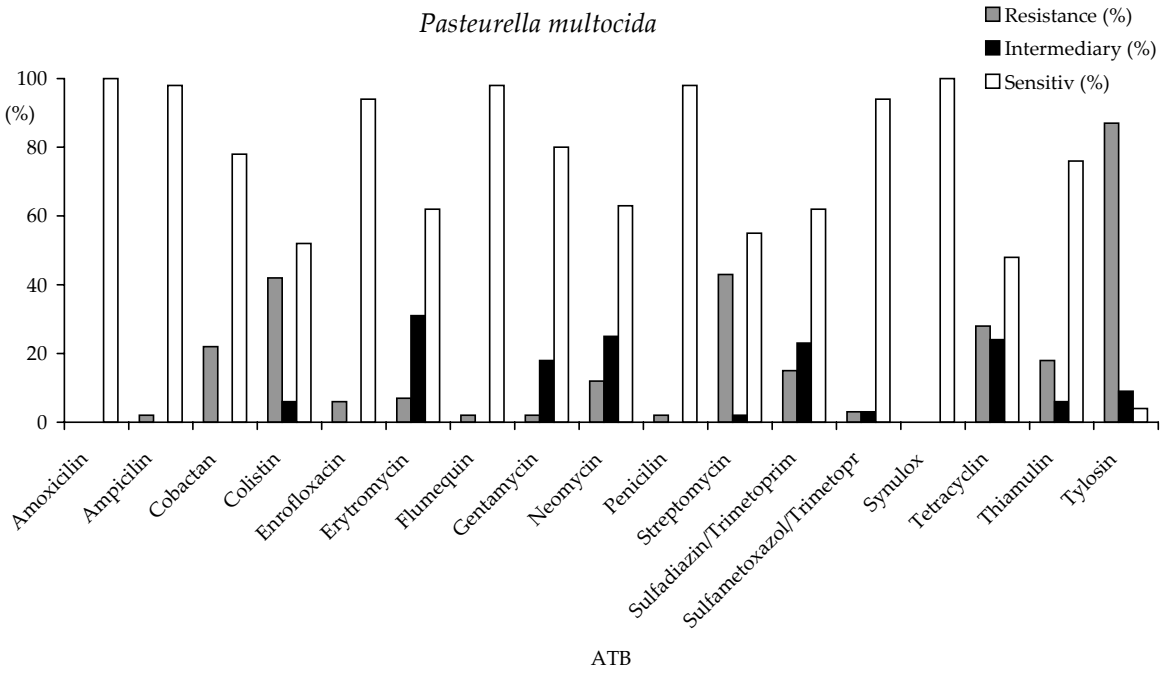

Figure 4. The results of antibiotic resistance testing of *Pasteurella multocida* strains isolated in course of years 2000–2001 (90 isolates)

ance testing of which is technically impossible. A relatively interesting seems to be the usage of sulphametoxazol potentiated by trimetoprime or gentamycin with its also relatively high sensitivity.

The occurrence of antibiotic resistant strains, resulting often from inconsiderate drug use, poses a serious hazard for the development of the epizootological situation and the current state in Slovakia

does not differ from that in other countries. Drug resistance of *A. pleuropneumoniae* is a world-wide problem which veterinary practitioners face when deciding on the treatment of acute porcine pleuropneumonia and/or preparing control programmes for large swine herds. The prevalence of drug resistant strains depends on geographical position, time of isolation, and drug used up to now. Nevertheless, more or less resistant strains occur world-wide (Wasteson *et al*., 1996). Antibiotic therapy is effective in clinically affected animals only in the initial phase of the disease when it can reduce mortality (Taylor, 1999). However, the use of antibiotics often does not eliminate the entire infection, and *A. pleuropneumoniae* may still be shed (Willson and Osborne, 1985). Herd depopulation is the most radical alternative to eliminate a disease outbreak. It consist of removing all animals from the farm site repopulating with animals from disease-free herds. In cases where there is a high prevalence of seropositive pigs in the herd, depopulation may be the only effective method of treatment (Nicolet, 1992).

On the basis of these results it is possible to state, on one hand, a high effectiveness of PCR used as an examination method in the diagnosis of pleuropneumonia-like organism and, on the other hand, the fact that *M. hyopneumoniae* plays a great role in the respiratory diseases in pig breedings of northern districts of Slovakia altogether with *P. multocida* that, as a secondary agent, enters running infection process. The result of this is *P. multocida's* most common finding when the primary agent has already been suppressed. *A. pleuropneumoniae* is in principle important as a pathogenic agent closely related to unsuitable zoohygienic breeding parameters.

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