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PHENOTYPIC AND GENOTYPIC CHARACTERISATION OF *PASTEURELLA MULTOCIDA* STRAINS ISOLATED FROM PIGS IN POLAND

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Abstract

A total of 319 *Pasteurella multocida* (*Pm*) strains isolated from pigs in Poland were examined. Phenotypic characterisation included: biochemical tests (to determine species, subspecies, and biovar), capsular typing, and antimicrobial susceptibility. Genotypic characterisation included detection of the *toxA* gene by PCR. All tested *Pm* strains were classified as *Pm* subsp. *multocida*: 87.2% biovar 3, 10.7%-2 and 0.9%-12. One strain was classified as biovar 1. Three strains of *Pm* did not suit any of the biovars. Using capsular typing methods, 77% of *Pm* strains isolated from nasal swabs belonged to type D and 33% to type A. Among *Pm* strains isolated from internal organs, 59.5% belonged to type A and 40.5% to type D. All the isolates showed a high susceptibility to β-lactams: ampicillin and amoxicilin with clavulonic acid (97.8%), penicillin (86.7%), doxicilline (100%), oxytetracycline (97.8%), and tetracycline (93.2%). It was found that all strains were susceptible to norfloxacin, 97.8% to enrofloxacin, and 95.6% to SxT. 24.4% and 15.6% of the strains were resistant to linco-spectin and tiamulin, respectively. The presence of *toxA* gene was confirmed by PCR in 20.8% of the strains isolated from nasal swabs and 29.1% of isolates from internal organs.

Key words: swine, *Pasteurella multocida*, capsular type, antimicrobial susceptibility, dermonecrotoxin, PCR.

Pasteurella multocida (*Pm*) is a worldwide spread pathogen of mammals' and birds' respiratory tract. It was isolated from cats, dogs, cattle, horses, sheep, poultry, rodents, monkeys, rabbits, lions, panthers, lynx, buffalo, and humans (8, 11, 12). *Pasteurella multocida* strains are also an important pathogen of swine. The strains with a high level of pathogenicity can be responsible for progressive atrophic rhinitis in pigs (18).

Pasteurella multocida is a non-motile, Gram-negative, non-haemolytic coccoid bacterium. It grows well on the most enriched media. *Pasteurella multocida* strains do not produce spores, whereas many isolates produce capsule, which plays an important role in the pathogenicity of *Pm*.

The classification of the genus *Pasteurella* has changed over time. Mutters *et al*. (24), divided *Pasteurella* genus into subsp. *multocida*, subsp. *septica*, and subsp. *gallicida*, based on DNA hybridisation results. Bisgaard *et al.* (3) determined the subspecies by their carbohydrate fermentation patterns. Based on the diversity to fermentation of different carbohydrates, Fegan *et al.* (13) described 10 biovars among *Pm* strains, and later Blackall *et al.* (4) added four other biovars to the classification system (37).

On the basis of the differences in polysaccharide antigens of the *Pm* capsule, five capsular types were established (A, B, C, D, F).

The methods of serotyping of *Pm* also have changed over time. For capsular serotyping, Carter (6) used indirect haemagglutination test. Heddleston (22) used the gel diffusion precipitation test and distinguished 16 serotypes. Townsend *et al.* (36) developed PCR for capsular typing of *Pm* strains. To confirm capsular type of the strains, the guidelines provided by the OIE has recommended acriflavine and hyaluronidase tests (27).

Progressive atrophic rhinitis caused by *Pm* and *Bordetella bronchiseptica* is an important disease from the economic point of view. A major pathogenicity factor of *Pm* strains is dermonecrotoxin (DNT) (1, 9). Dermonecrotoxin, a 143-146 kDa heatsensitive protein, is encoded by *toxA* gene. Toxigenic strains can be distinguished *in vitro* by their toxigenicity for tissue culture, or *in vivo* by experimental infection of different species, including mice and guinea-pigs (10). DNT of *Pm* strains can also be detected by ELISA and PCR.

Diversity among *Pm* strains suggests that classical bacteriological methods supported by molecular techniques are essential for the identification and characterisation of *Pm* strains.

The study has been focused on phenotypic and genotypic characterisation of Polish *Pm* strains isolated from pigs.

Material and Methods

***Pasteurella multocida* strains.** A total of 319 *Pm* strains isolated from pigs were analysed. One hundred and seventy-eight *Pm* strains were isolated from nasal swabs and 141 strains were obtained from the lungs (115), heart (14), kidneys (4), liver (4), lymph nodes (3), and spleen (1). Nasal swabs were obtained from 106 pigs' farms located in 13 Polish provinces between 2007 and 2011: Wielkopolska (35), Śląsk (17), Kujawsko-Pomorskie (13), Mazowieckie (10), Zachodnio-Pomorskie (8), Lubelskie (7), Warmińsko-Mazurskie (4), Łódzkie (3), Podlaskie (3), Lubuskie (2), Świętokrzyskie (2), Dolnośląskie (1), and Małopolskie (1). All isolates showing typical morphology of colonies and biochemical features of *Pm*.

Culture conditions. Bacterial cultures were cultivated on 5% horse blood agar at 37°C for 24 h, according to standard procedure.

Biochemical characterisation and classification. Identification of *Pm* isolates was done by biochemical tests: catalase, oxidase, ornithine decarboxylase (ODC), urease activity and nitrate reduction. Carbohydrate fermentation was determined using the following carbohydrates: trehalose, galactose, xylose, arabinose, mannitol, lactose, sorbitol, maltose, and glucose.

Species were identified by testing nitrate reduction, urease activity, and glucose fermentation. Subspecies were defined on the basis of sorbitol fermentation. Biovars were determined by trehalose, galactose, xylose, arabinose, mannitol, lactose, sorbitol, maltose, and glucose fermentation and by testing the ODC activity.

Capsular serotyping. Capsular serotyping was performed according to Carter method. Type D strains were identified by their ability of agglutination in the presence of acriflavine solution. Type A strains were characterised by inhibition of capsular formation by hyaluronidase.

Antimicrobial susceptibility. Isolates of *Pm* were tested for their sensitivity to a panel of fifteen antibiotics by the disc diffusion method, following the recommendations and criteria provided by the National Committee for Clinical Laboratory Standards. It consists of ampicillin (10 µg/mL, Oxoid), amoxicillin with clavulonic acid (30 µg/mL, Oxoid), enrofloxacin (5 µg/mL, Oxoid), norfloxacin (10 µg/mL, Oxoid), doxicilline (30 µg/mL, Oxoid), ceftiofur (30 µg/mL, Oxoid), tetracycline (30 µg/mL, Oxoid), neomycin (30 µg/mL, Oxoid), oxytetracycline (30 µg/mL, Oxoid), gentamicine (10 µg/mL, Oxoid), penicillin (10 µg/mL, Oxoid), linco-spectin (109 µg/mL, Oxoid), SxT (1.25+23.75 µg/mL, BioMerieux), tiamulin (30 µg/mL, Rosco), and tylosin (30 µg/mL, Mast Group Ltd.).

Presence of DNT *Pm*. Primers, which amplify a 501bp fragment of the *toxA* gene of *Pm*, designed by Register *et al.* (31) were used. PCR was performed in 25 µl mixture containing 10xPCR Gold Buffer, 25 mM $MgCl_2$ solution, polymerase AmpliTaq Gold 5U/ml, 10 mM dNTPs (Fermentas), and 20 pM each of primer and water free from DNse and RNase. The reaction mixture was subjected to an amplification regimen consisting of an initial denaturation step (95°C, 10 min) followed by 40 cycles at 94°C (30 s), 56°C (30 s), and 72°C (30 s) and final elongation at 72°C (7 min), with the use of a T3-Biometra thermocycler.

Products obtained in PCR were separated by electrophoresis in 2% agarose gel with ethidium bromide in concentration of 1 µg/mL. The electrophoresis was done in 1xTAE buffer, at 350 mA. Into each well, 10 µl of reaction mixture and 2 µl of loading buffer 6xDNA Loading Dye (Fermentas) were inserted. The molecular weight of the obtained products was determined by comparison to the molecular weight of marker GeneRuler™ 100 bp DNA Ladder Plus (Fermentas). The agarose gels were photographed under UV light using EC3 Chemi HR 410 Imaging System (UK).

Results

Phenotypic characterisation. All *Pm* strains showed typical growth on agar plates; they were G (-) and were producing catalase. All isolates were oxidase positive, nitrate negative, lacked urease and haemolytic acivity, and all failed to grow on MacConkey agar. Additionally, the field isolates of *Pm* showed ornithine decarboxylase activity. Based on glucose, lactose, sorbitol, mannitol, trehalose, maltose, xylose, and arabinose fermentation all species were classified as *Pm* subsp. *multocida*. 87.2% of the species were classified as biovar 3, 10.7% as biovar 2, and 0.9% strains as biovar 12 (*Pm* subsp. *multocida* lactose positive). One strain was classified as biovar 1. Three strains could not be classified into any of the biovars. Table 1 shows the results of biovar classification based on the sugar fermentation.

Determination of capsule type. It was estimated that 77% of *Pm* isolates from nasal swabs belonged to capsule type D and 33% of the isolates belonged to type A. 59.5% of *Pm* strains isolated from internal organs belonged to type A and 40.5% to type D.

Antimicrobial susceptibility. All *Pm* isolates showed a high susceptibility to β-lactams: ampicillin and amoxicilin with clavulonic acid (97.8%), penicillin (86.7%); tetracyclines: doxicilline, oxytetracycline, and tetracycline (100%, 97.8%, and 93.2%, respectively). It was found that all strains were susceptible to norfloxacin, 97.8% to enrofloxacin, and 95.6% to SxT. 24.4% and 15.6% of the strains were resistant to linco-spectin and tiamulin, respectively. Fig. 1 shows antimicrobial susceptibility results of *Pm* strains.

Molecular characterisation. Thirty-seven from 178 (20.8%) *Pm* isolates from nasal swabs were positive for the presence of *toxA* gene and produced the 501bp product by specific PCR. Fig. 2 shows gel-

electrophoresis of PCR products obtained from DNA of *Pm* strains.

The *toxA* gene was detected in 28.7% of *Pm* isolates obtained from the lungs, 42.9% from heart, 25% from kidneys and liver. *Pasteurella multocida* isolates obtained from the lymph node and spleen were negative for *toxA* gene.

Table 1
Profile of sugar fermentation Polish *Pm* isolates

Fermentation									%	Biovar
Trehalose	Galactose	Xylose	Arabinose	Mannitol	Lactose	Sorbitol	Maltose	Glucose		
-	+	+	-	+	-	+	-	+	87.2	3
+	+	+	-	+	-	+	-	+	10.7	2
-	+	+	-	+	+	+	-	+	0.9	12
+	+	+	-	+	+	+	-	+	0.3	1
-	+	-	-	+	-	+	-	+	0.3	-
+	+	-	-	+	-	+	-	+	0.3	-
+	+	+	-	+	-	+	-	-	0.3	-

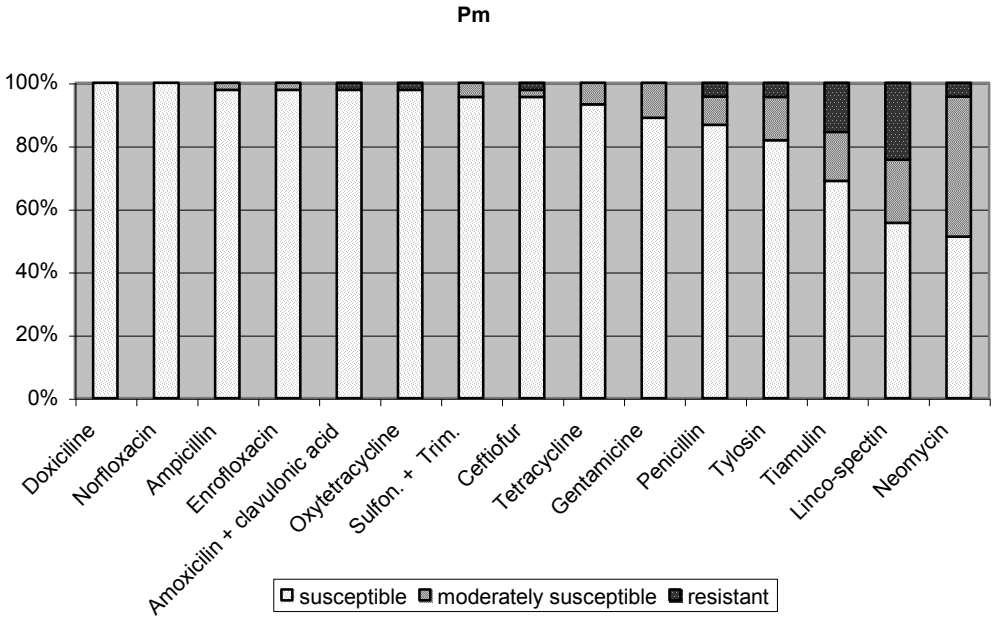


Fig. 1. Antimicrobial susceptibility of *Pm* strains

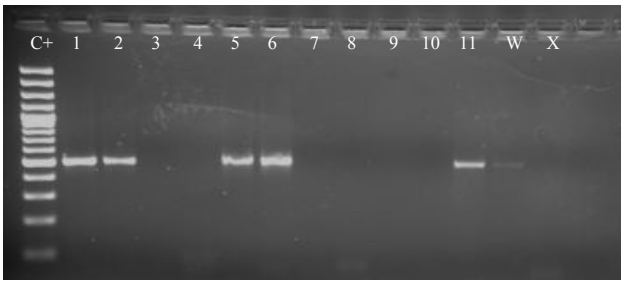


Fig. 2. Electrophoresis of PCR products. M-DNA molecular weight marker GeneRuler™ 100 bp DNA Ladder Plus, C+ - positive control DNT+*Pm*, 1-11 tested samples, W-water (negative control), X-mix (negative control)

Discussion

Based on biochemical test results and colony morphology, all species were classified as *Pm*. All tested strains were classified as *Pm* subsp. *multocida.* The ability to sorbitol fermentation by all tested strains excluded their affiliation to *Pm* subsp. *septica* (24). *Pasteurella multocida* subsp. *gallicida* shows the ability of arabinose fermentation (13) and they are mainly isolated from poultry. Fussing *et al.* (14) classified 133 DNT+*Pm* strains to subspecies relying on a previous study by Nielsens *et al.* (26), which showed that *Pm* strains with dermonecrotoxin belong to *Pm* subsp. *multocida*. Blackall *et al.* (4) made phenotypic characterisation of 150 Australian *Pm* strains according to Mutters *et al.* (24) guidelines and found that 91.3% of *Pm* strains belonged to *Pm* subsp. *multocida* and 8.7% to *Pm* subsp. *gallicida*. Blackall *et al.* (4) classified those strains into 14 biovars, according to their sugar profile. 72.9% *Pm* subsp. *multocida* strains belonged to biovar 3, 13.8% to biovar 2, 6.5% to biovar 12 (*Pm* subsp. *multocida* lactose positive). All *Pm* subsp. *gallicida* strains belonged to biovar 8. Four *Pm* isolates were classified to biovars 13 and 14. Similar results to Blackall *et al.* (4) were obtained by Bowles *et al.* (5). He classified 103 from 107 tested *Pm* strains as *Pm* subsp. *multocida* and 69.9% of them belonged to biovar 3, whereas 14.5% to biovar 2. Similar results were also obtained in population of Hungarian *Pm* strains isolated from pigs. 67.7% *Pm* subsp. *multocida* belonged to biovar 3, 16.5% to biovar 2, and 7.9% to biovar 12 (35). The study showed that 87.2% of tested *Pm* subsp. *multocida* strains belonged to biovar 3, 10.7% to biovar 2, and 0.9% to biovar 12.

To confirm the capsular type of *Pm* strains, acriflavine and hyaluronidase tests were used. It was demonstrated that Polish population of *Pm* strains from AR cases belonged mainly to type D (77%).

Lariviere *et al.* (20) reported that in Canada 53% *Pm* strains obtained from nasal swabs belonged to capsular type D and 47% to type A. The authors noted that capsular antigens of the tested strains did not correlate with clinical signs of AR. *Pasteurella multocida* strains isolated from nasal swabs in Japan were also represented mostly by capsule type D (68%) (33). Similar results were shown by Lichtensteiner *et al.* (21) and Fussing *et al.* (14). The percentage of *Pm* strains with D capsular type was 60% and 58.8%, respectively. The highest percentage (82.3%) of *Pm* strains with type D from AR case was demonstrated in Australia (15).

Analysis of capsular type by multiplex PCR showed that type D was represented by 57.1%, 60%, 70.6% and 76% *Pm* strains in Czech Republic (16), Hungary (37), England (7), and Germany (2), respectively.

Except determination of capsular type of *Pm* strains, it is important to define their ability to DNT production. Gardner *et al.* (15) found DNT in 64.6% of *Pm* strains belonging to type D and in 5.9% belonging to type A. Lichtensteiger *et al.* (21) reached similar results referring to *Pm* strains with type D, but the percentage of *Pm* strains of type A with DNT obtained in his study was higher (25%). Results obtained by Nagai *et al.* (25), Davies *et al.* (8), and Sawata *et al.* (33) showed that 57.5%, 58.3%, and 59.8% *Pm* strains of type D had DNT, respectively.

In Germany, Ewers *et al*. (12) found *toxA* gene in 36% of type A and 30% of type D *Pm* strains. In New Zealand, Jamaludin *et al*. (17) found 92% of type A and only 2% of type D strains among 48 porcine isolates but *toxA* gene had been detected in none of them. Jamaludin *et al.* (17) and Varga *et al.* (37) explained this phenomenon by the theory of horizontal gene transfer, which suggests that *toxA* gene is absent in geographically isolated countries. Varga *et al.* (37) showed that in Hungary 60% of 146 tested *Pm* strains isolated belonged to type D and 38% to type A, but *toxA* was more common in type A isolates (41%). Varga and Jamaludin support the theory concerning regional distribution of *Pm* strains with *toxA* gene. Davies *et al.* (7) showed that 9% of *Pm* strains isolated in England and Wales from AR cases had *toxA*, and mostly belonged to type D.

Analysis of antibiotic susceptibility of *Pm* strains demonstrated that tested strains were sensitive to ampicillin, doxicilline, norfloxacin, enrofloxacin, amoxicillin with clavulonic acid, oxytetracycline, and SxT. The results are similar to data received by Pejsak *et al*. (28, 29). High susceptibility of the strains to ceftiofur was demonstrated. The efficiency of this antibiotic was emphasised by Post *et al.* (30), Yoshimura *et al.* (38), and Salmon *et al.* (32).

Comparing the susceptibility of Polish *Pm* strains with Korean *Pm* strains isolated from swine and cattle (34), it could be stated that our strains were less resistant to enrofloxacin, ceftiofur, ampicillin, tylosin, and SxT. The difference in the susceptibility ranged from 19.8% to 59.6%.

Studies of Millan *et al.* (23) and Tang *et al*. (35) underlined the occurrence of multiresistance in population of *Pm* strains. The first authors (23) showed that 2.5% of *Pm* strains isolated in Spain between 2002 and 2005 were resistant to ampicillin and tetracycline, simultaneously. In turn, Tang *et al.* (35) demonstrated that 93.1% of Chinese *Pm* strains isolated from swine were resistant to 3-10 antibiotics. *Pm* strains were resistant to gentamicine (13.7%), tetracicline (58%), and SxT (74.2%), while Polish isolates were sensitive to these antibiotics. Polish *Pm* strains were also multiresistant, 20% and 13.5% of them were resistant to 2 and 3 antibiotics, respectively. It was also demonstrated by Kuczkowski *et al.* (19), who discovered that every fourteen Polish *Pm* strains isolated from geese or turkeys were resistant to at least six antibiotics.

Tang *et al.* (35) observed that multiresistance was more common in population of *Pm* with capsular type D. There is no correlation between the ability of *Pm* strains to DNT production and their multiresistance.

In summary, the study showed that all *Pm* strains isolated in Poland belonged to subsp. *multocida* and the majority of them represented biovar 3. The study also showed the difference in capsular type of *Pm* strains

recovered from nasal swabs and from internal organs. The most strains from nasal swabs belonged to type D (77%), while the majority of strains from internal organs represented capsular type A (59.5%). *ToxA* gene was present in 20.8% of *Pm* strains from nasal swabs and in 29.1% of isolates from internal organs. All *Pm* isolates showed a high susceptibility to most tested antibiotics particularly to β-lactams and tetracyclines.

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