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Molecular characterization of *Pasteurella multocida* isolates from swine lungs by *Randomly Amplified Polymorphic DNA*

Caracterização molecular de isolados de *Pasteurella multocida* de pulmões de suínos através da técnica *Randonly Amplified Polymorphic DNA*

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ABSTRACT

*Swine respiratory diseases such as atrophic rhinitis and bronchopneumonia caused by **Pasteurella (P.) multocida** cause important economic losses to the modern swine industry. The purpose of this study was to characterize **P. multocida** strains isolated from swine lungs by RAPD (**Randomly Amplified Polymorphic DNA**) to demonstrate their genetic diversity. Ninety-four samples of fragments from lungs with pneumonia and sixty one samples without pneumonia were collected in slaughterhouses in Mato Grosso during the period from December 2009 to March 2010. Clinical cases in 2012 and 2013 were also included in this study. Among the lung fragments with macroscopic lesions, without macroscopic lesions and clinical samples, 40.42%, 4.49% and 100% were positive for **P. multocida**, respectively. Bacterial identification culturing was confirmed by PCR (polymerase chain reaction) by means of the amplification of the gene kmt1. RAPD technique was performed for 46 isolates, and in every isolate, a total of 7 to 11 amplification bands were detected, composed of 8 clusters based on genetic similarity. Thus, treatment, control and preventive measures should consider the genetic diversity of **P. multocida** populations in swine herds in order to improve the development of new protocols to produce antimicrobials and vaccines.*

Key words: *pulmonary pasteurellosis, swine, pneumonia, **Pasteurella multocida**, RAPD.*

RESUMO

*As doenças respiratórias suínas como a rinite atrófica e broncopneumonia, associada a **Pasteurella (P.) multocida** causam importantes perdas econômicas na suinocultura moderna. O objetivo deste trabalho foi caracterizar isolados de **P. multocida** de pulmão suíno através do **Randomly Amplified Polymorphic DNA** (RAPD) para demonstrar a diversidade genética. Noventa e quatro amostras de fragmentos de pulmões com lesões de pneumonia e sessenta e uma amostras sem lesão foram coletadas em frigoríficos no Estado do Mato Grosso, durante o período de dezembro de 2009 a março de 2010. Amostra de casos clínicos ocorridos em 2012 e 2013 também foram inlcuídos. Amostras de pulmões com lesões macroscópicas, sem lesões macroscópicas e amostras clínicas apresentaram presença de 40,42%, 4,49% e 100% de isolamento para **P. multocida**, respectivamente. Os isolados foram todos confirmados através da PCR (**Polymerase Chain Reaction**) pela amplificação do gene kmt1. A técnica de RAPD foi realizada em 46 amostras e em cada isolado foi detectado 7 a 11 bandas, que foram agrupadas em 8 grupos baseados em suas similaridades genéticas. Dessa forma, tratamento, controle e medidas preventivas deveriam considerar a diversidade genética da população de **P. multocida** em rebanhos suínos para melhorar o desenvolvimento de novos protocolos para produção de antimicrobianos e vacinas.*

Palavras-chave: *pasteurelose pulmonar, suíno, pneumonia, **Pasteurella multocida**, RAPD.*

INTRODUCTION

Respiratory diseases caused by ***Pasteurella (P.) multocida*** in swine, such as atrophic rhinitis and bronchopneumonia, are important in the swine industry and responsible for significant losses in modern swine production (ZHAO et al., 1992; BOROWSKY, 2006). The occurrence of ***P. multocida*** in other species has also been reported, such as pasteurellosis ('snuffles') in rabbits, fowl cholera in poultry, hemorrhagic septicemia in ruminants and animal scratches and bites in humans (ARUMUGAM et al., 2011; ASHRAF et al., 2011; LÓPEZ et al., 2013; WILSON & HO, 2013 RAVAL et al., 2014). Pulmonary pasteurellosis is usually associated with

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other pathogens and principally affects animals in the final stage of enzootic pneumonia caused by ***Mycoplasma hyopneumoniae*** or porcine respiratory disease complex (PRDC) (PIJOAN, 2006). However, studies have experimentally reproduced respiratory disease in pigs by inoculating ***P. multocida*** in Specific Pathogen Free (SPF) animals (KICH et al., 2007).

P. multocida is a Gram-negative bacterium grouped into five capsular serogroups (A, B, D, E and F) and is further classified into 16 somatic serotypes according to the lipopolysaccharide antigen (HEDDLESTON, 1972; PIJOAN, 2006; BOROWSKY et al., 2007). In pigs, serotypes A and D are associated with respiratory disease and are widely distributed among herds (BOROWSKY et al., 2007). Serogroup A is the serotype most associated with pneumonia (PIJOAN, 2006), although studies also show an increase in serogroup D isolated from pig lugs in slaughterhouses (JORDAN, 2006; MORES, 2006).

P. multocida has been characterized by phenotypic and genotypic methods. Phenotypic methods include somatic and capsular serotyping and physiological and outer membrane profiles, while endonuclease analysis, ribotyping, pulsed field gel electrophoresis, fingerprinting PCR, random amplified polymorphic DNA (RAPD) and multiple locus sequencing typing (MLST) represent the genotypic methods (HEDDLESTON et al., 1972; WILLIAMS et al., 1990; DZIVA et al., 2001; DZIVA et al., 2004).

Genetic diversity in isolates of ***P. multocida*** is characterized by associating genotypes with specific hosts such as cattle and poultry, as well as with geographic locations (AL-HADDAWI et al., 1999; TAYLOR et al., 2010; MOHAMED & MAGEED, 2014)**.** The purpose of this study is to characterize the genetic diversity of ***P. multocida*** isolated from swine lungs by RAPD.

MATERIALS AND METHODS

Lungs condemned with pneumonia and without gross lesions were collected from 94 and 61 finishing pigs, respectively, during December 2009 to March 2010, in a slaughterhouse in Mato Grosso, Brazil. The animals were 120 to 160 days old. Additionally, five clinical samples from farms that had respiratory problems were received by our laboratory in 2012 and 2013. Samples were from seven cities in Mato Grosso and one county in Mato Grosso do Sul (Table 1). The gross lesions in the lungs with pneumonia that were included in this study were consolidation, fibrin deposition on the pleura, pleurisy and/or adhesion. Samples without microscopic and macroscopic lesions were included in the control group. Tissue samples from the lungs were taken to be analyzed under refrigeration, and a fragment was kept at -20°C until further molecular tests were performed.

Tissue samples for the isolation of ***P. multocida*** were macerated and plated on 5% sheep blood agar (Sigma-Aldrich, St Louis, MO, USA) and MacConkey (Sigma-Aldrich, St Louis, MO, USA) agar, incubated at 37°C for 48 hours in aerobiosis, and biochemically characterized (QUINN et al., 1994). Samples that contained Gram-negative coccobacilli that did not grow in MacConkey (Sigma-Aldrich, St Louis, MO, USA) agar were subjected to biochemical tests to confirm that they were ***P. multocida***. Confirmed samples were catalase positive, oxidase, urease, glucose, sucrose and mannitol positive and negative for lactose and maltose and fermented only on the slant of the tube in TSI (triple sugar iron) (HiMedia Laboratories, Vadhani Industrial Estate, Mumbai, India) medium.

The isolates classified as ***P. multocida*** by the biochemical tests were cultivated in 3mL of BHI (brain heart infusion) (Oxoid, Basingstoke, Hants, England) broth for 18 hours at 37°C under constant agitation. DNA extraction followed, using a phenol/chloroform method according to SAMBROOK & RUSSEL (2001). For each sample, 1mL of BHI (Oxoid, Basingstoke, Hants, England) broth was collected and added to a 2mL microtube with 1mL of lysis buffer (100 mM NaCl, 10mM Tris pH 8.0, 25mM EDTA, 0.5% SDS, and 0.1mg mL^{-1} of proteinase K). Tubes were vortexed and incubated for 3 hours at 50°C, and phenol/chloroform extraction followed. Aqueous phase was transferred to a 1.5mL microtube, and the DNA was precipitated with 0.3M sodium acetate and one volume of isopropanol at -20°C overnight. DNA was collected by centrifugation at 16.000g for 15 minutes, and the supernatant was discarded. The pellet was washed with 500µL 70% ethanol and suspended in 100µL ultra-pure water. PCR (Polymerase Chain Reaction) based on the ***P. multocida*** gene kmt1 was performed to confirm the isolates as ***P. multocida***, as described by TOWNSEND et al. (2001). Reaction mix contained 50ng DNA at a final volume of 20µL, Taq Buffer solution 1X (10mM Tris-HCl pH 8.3, 50mM KCl, 2.5mM $MgCl_2$), 250µM each dNTP, 3mM $MgCl_2$, 1U of Taq DNA polymerase (Life Technologies Corporation, Van Allen Way Carlsbad, CA, USA) and 1µM each of primers KMT1T7 (5'ATCCGCTATTTACCCAGTGG3') and KMT1SP6 (5'GCTGTAAACGAACTCGCCAC3'). In the reaction profile, 95°C was maintained for

Table 1 - Region, serogroup, RAPD cluster, Sequence type (ST) and Clonal Complex (CC) of ***P. multocida*** isolates.

Isolate	City	Kmt1	Sorogroup	RAPD	ST	CC
M810-10	Sorriso	+	A	I	NC*	NC
M716-10	Diamantino	+	A	I	NC	NC
M699-10	Diamantino	+	D	I	NC	NC
M808-10	Sorriso	+	A	I	NC	NC
M809-10	Sorriso	+	A	I	NC	NC
M166-10	Sorriso	+	A	I	197	NC
M714-10	Diamantino	+	A	I	NC	NC
M713-10	Pedra Preta	+	A	I	NC	NC
M708-10	Pedra Preta	+	A	I	NC	NC
M704-10	Diamantino	+	A	I	NC	NC
M715-10	Diamantino	+	A	I	NC	NC
M717-10	Diamantino	+	A	I	NC	NC
M705-10	Diamantino	+	A	I	NC	NC
M722-10	Diamantino	+	A	I	NC	NC
M719-10	Diamantino	+	A	I	NC	NC
M412-13	Jaciara	+	A	II	NC	NC
M700-10	Diamantino	+	D	II	NC	NC
M698-10	Diamantino	+	D/A	II	NC	NC
M813-10	Diamantino	+	A	II	NC	NC
M814-10	Diamantino	+	A	II	NC	NC
M718-10	Diamantino	+	A	II	NC	NC
M817-10	Campo Verde	+	A	II	NC	NC
M815-10	Campo Verde	+	D	II	NC	NC
MTF06-02	Nova Mutum	+	A	II	NC	NC
M151-10	Campo Grande	+	A	II	27	ST74
M150-10	Campo Grande	+	A	II	27	ST74
M171-10	Sorriso	+	A	II	27	ST74
M703-10	Itiquira	+	A	II	NC	NC
M413-10	Jaciara	+	A	II	NC	NC
M807-10	Sorriso	+	A	III	NC	NC
MTG05-01	Nova Mutum	+	A	IV	NC	NC
M155-10	Campo Grande	+	A	IV	27	ST74
MTF01-01	Nova Mutum	+	A	IV	NC	NC
M173-10	Sorriso	+	A	IV	27	ST74
M163-10	Sorriso	+	A	V	NC	NC
M160-10	Campo Grande	+	A	V	190	NC
M162-10	Campo Grande	+	A	V	192	ST74
M154-10	Campo Grande	+	A	V	27	ST74
M167-10	Sorriso	+	A	V	196	ST13
M161-10	Campo Grande	+	A	V	191	ST74
M153-10	Campo Grande	+	A	V	27	ST74
M164-10	Sorriso	+	A	V	196	ST13
M175-10	Pedra Preta	+	A	VI	193	NC
M142-10	Nova Mutum	+	A	VI	27	ST74
M225-08	Santa Rita do Trivelato	+	A	VII	198	NC
M135-10	Sorriso	+	A	VIII	13	ST13

*NC = not classified.

5min, followed by 30 cycles of denaturation at 95°C for 30sec, annealing at 50°C for 30sec, extension at 72°C for 30sec and final extension at 72°C for 5min. A 460bp DNA fragment was expected. DNA and amplification product quality and integrity were analyzed by electrophoresis in 1.0% agarose, stained

with Gel Red™ (Biotium®, Hayward, CA, USA) at 10 volts per centimeter and observed in ChemiDoc™ XRS+ (Bio-rad Laboratories, Inc., Hercules, CA, USA) using the software Image Lab™ (Bio-rad Laboratories, Inc., Hercules, CA, USA). The 100 base pair DNA Ladder™ (Life Technologies Corporation, Van Allen Way Carlsbad, CA, USA) was used as a molecular weight marker.

Isolates considered positive for the kmt1 gene by PCR were subjected to the RAPD technique with the M13 (5'GAGGGTGGCGGTTCT3') primer previously described by VASSART et al. (1987). Reaction mix contained 50ng DNA at a final volume of 25µL, Taq Buffer solution 1X (10mM Tris-HCl pH 8.3, 50mM KCl, 2.5mM $MgCl_2$), 200µM each dNTP, 3.5mM $MgCl_2$, 2.5U of Taq DNA polymerase (Life Technologies Corporation, Van Allen Way Carlsbad, CA, USA) and 1µM of primer M13. In the reaction profile, 95°C was maintained for 3min, followed by 30 cycles of denaturation at 95°C for 30sec, annealing at 44°C for 58sec, extension at 72°C for 70sec and final extension at 72°C for 7min. Amplification products were analyzed by electrophoresis in 2.0% agarose, stained with Gel Red™ (Biotium®, Hayward, CA, USA) at 10 volts per centimeter and observed in ChemiDoc™ XRS+ (Bio-rad Laboratories, Inc., Hercules, CA, USA) using the software Image Lab™ (Bio-rad Laboratories, Inc., Hercules, CA, USA). The 100 base pair DNA Ladder™ (Life Technologies Corporation, Van Allen Way Carlsbad, CA, USA) was used as a molecular weight marker.

In each amplification reaction, the presence (1) or absence (0) of amplicons generated binary matrix values. The DICE coefficient was used to estimate the genetic similarity of ***P. multocida*** isolates, and a dendrogram was obtained by the method of UPGMA (unweighted pair group arithmetic average) with a Bootstrap of 100, using the program PyElph 1.4 (PAVEL & VASILE, 2012).

Isolates were compared with the genotypes obtained by MLST as described by SILVA et al. (2012); the presence of the genes adk, est, pmi, zwf, mdh, gdh and pgi was detected as previously described by SUBAAHARAN et al. (2010).

RESULTS AND DISCUSSION

Among 94 samples with gross lesions, 61 without lesions and 5 clinical cases, there were 38 (40.42%), 3 (4.9%) and 5 (100%) samples positive for ***P. multocida,*** respectively. The results were confirmed by amplification of the gene kmt1 via PCR. Serogroup A was the most prevalent (91.3% of the isolates), and serogroup D was present in 8.7% of the isolates. Seventeen isolates were subjected to PCR and genotyping by sequencing 7 genes for the MLST technique (SILVA et al., 2012) (Table 1).

DNA fragment patterns with 7 to 11 bands were generated with the M13 primer and varied in molecular weight from 100bp to 1500bp. Banding pattern observed with UPGMA analysis revealed that 8 major clusters were formed (I to VIII), illustrating genetic relationships among the isolates. The most frequent clusters were I (32.6%), II (30.4%), V (17.4%), IV (8.7%) and VI (4.3%). Clusters III, VII and VIII occurred in only one isolate (Figure 1). RAPD is a method that has been used to compare different bacterial strains for epidemiologic studies and to show diversity among strains (AL-HADDAWI et al., 1999), with better results than other techniques such as SDS-PAGE (MOHAMED & MAGEED, 2014).

TAYLOR et al. (2010) used the same primer (M13) to characterize 41 isolates of ***P. multocida*** isolated from cattle with respiratory diseases and found 13 clusters in which each isolate generated DNA fragment patterns containing 12 to 18 bands. In another study, LEE et al. (2012) characterized 47 isolates of ***P. multocida*** serogroup A from swine by RAPD with the same primer and found 12 clusters. These studies demonstrate that the M13 primer used for RAPD is useful for genetic differentiation of the isolates.

Isolates originated from nine cities; most isolates were from cluster II (77.7%), which was found in seven cities, followed by isolates from cluster I (33.3%) and cluster IV, (33.3%) found in three cities. Among the samples collected in the slaughterhouse, 40% (5/8) of isolates from Campo Grande were in cluster V, the isolates from Diamantino were in clusters I and II, and the isolates from Sorriso were in clusters I, II, III, IV, V and VIII. Isolates from Diamantino and Campo Grande showed greater genetic similarity, probably because the animals were from the same farm, while isolates from Sorriso showed greater genetic difference, probably because the animals were from farms that had a mixture of animals from different regions in the grow-finish phase and probably the indiscriminate use of antimicrobials in all farms becomes more frequent over time. The resistance of strains of ***P. multocida*** in China increased from 47.8% in 2003 to 97.1% in 2007 to more than 5 antibiotics (TANG et al., 2009).

Isolates of ***P. multocida*** serogroup D were in 2 clusters (I and II), and three of these isolates were in cluster II. In South Korea, all isolates of ***P.***

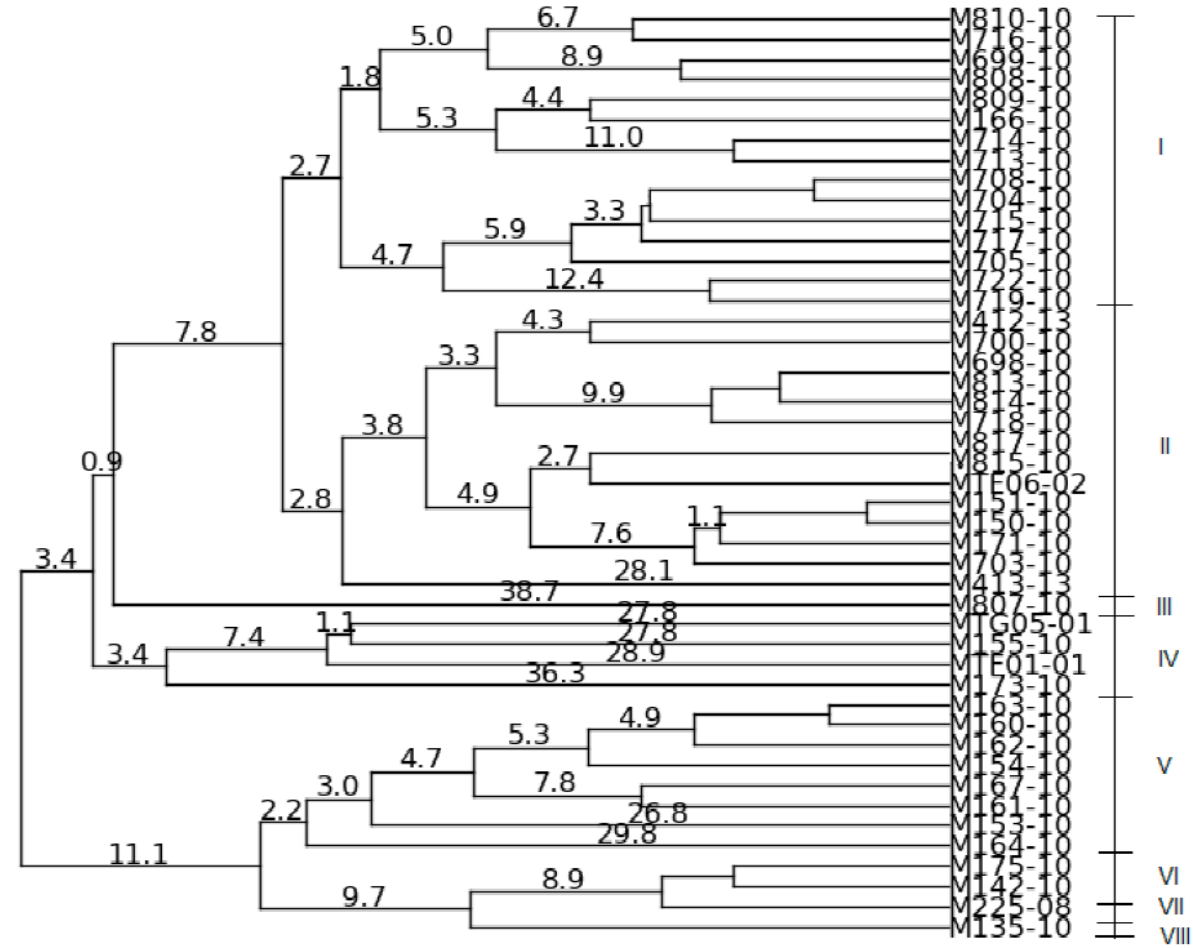

Figure 1 - Dendogram obtained by Dice coefficient for genetic dissimilarity between isolates using primer M13 generated by PyElph 1.4 software with UPGMA analyze, with a bootstrap of 100. The clusters I, II, III, IV, V, VI, VII and VIII were formed by 95% of similarity among the isolates.

multocida serogroup D were in the same cluster and showed 100% of similarity (LEE et al., 2012).

The MLST technique was performed on seventeen isolates, and nine sequence types (ST) were detected, whereas RAPD only detected seven (Table 1). Due to the differences in each technique (RAPD and MLST), comparison between the techniques and isolates is not possible (SACHSEA et al., 2014).

The clonal complex detected most frequently was ST74 (58.82%), followed by ST13 (17.6%), whereas ST74 isolates were found only in clusters II and IV, confirming that the RAPD technique has the most discriminatory power for genetic variation. Cluster V contained isolates from two complexes, ST13 from Sorriso and ST74 from Campo Grande. Most of the isolates from this study belong to ST74 and ST13, which was similar to swine in Norway (PORS et al., 2011), except that ST50 was absent in our study. These isolates are probably species-specific (HOTCHKISS et al., 2011) with the exception of ST13, which has also been identified in cattle.

In avian pasteurellosis, observation of multiple genotypes within an outbreak may hamper an autogenous vaccines because one genotype could not cross-protected other. In this case, an autogenous vaccine should include all relevant genotypes or serovars in the outbreak (SINGH et al., 2013).

CONCLUSION

P. multocida isolates from Mato Grosso and Mato Grosso do Sul occurred in 8 clusters as determined by the RAPD technique, demonstrating high genetic diversity. These results suggest that control measures, treatment and prevention should consider the genetic diversity of ***P. multocida*** in swine

in order to improve protocols for the development of antimicrobials and vaccines.

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