Acta Veterinaria Brasilica December 13 (2019) 224-227

Acta Veterinaria Brasilica

Journal homepage: https://periodicos.ufersa.edu.br/index.php/acta/index

Original Article

Evaluation of two DNA extraction techniques in the detection of *Mycoplasma hyopneumoniae* by PCR in lungs samples of pigs with and without pneumonia

Avaliação de duas técnicas de extração de DNA na detecção de Mycoplasma hyopneumoniae por PCR em amostras de pulmões de suínos com e sem lesão de pneumonia

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A R T I C L E I N F O

Article history
Received 16 June 2019
Accepted 23 September 2019

Keywords:
Mycoplasma hyopneumoniae
PCR
Enzootic Swine Pneumonia

A B S T R A C T

Swine Enzootic Pneumonia (PES) has as the primary etiologic agent the *Mycoplasma hyopneumoniae*. The diagnosis of PES involves clinical manifestations on farms, macroscopic lesions visualized in the slaughterhouse and laboratory techniques, such as isolation by culture, histopathology, immunohistochemistry, ELISA and other serological tests and PCR. This work aimed to establish the comparison between PCR results and typical macroscopic findings in lungs with PES and to evaluate the efficacy of PCR results with extraction by phenol-chloroform and commercial kit in fragments of swine lungs. The efficacy of PCR was evaluated by comparing the classic method of DNA extraction by phenol-chloroform and by commercial Kit. Fragments of lungs of 18 pigs with characteristic lesions of PES and 17 without lesions were subjected to DNA extraction and posterior PCR. The pulmonary lesions with PES were PCR-positive in 94.44%, compared to 76.47% of the non-PES lungs. Kappa concordance between macroscopic and PCR was 18%, which was not significant (P > 0.05). Of the 35 lung fragments processed by phenol-chloroform, 85.71% were positive by PCR, compared to 28.57% by the commercial kit. Kappa agreement between DNA extraction methods was 57%, which was statistically significant (p <0.05), but Odds ratio was 15 (CI 4.52 to 49.68), which means that the probability of PCR positivity by the phenol-chloroform method was 15 times higher than that by the commercial kit. Macroscopy and PCR results were discordant, with phenol-chloroform extraction being the best between both procedures.

Palavras-chave:
Mycoplasma hyopneumoniae
PCR
Pneumonia Enzoótica Suína

R E S U M O

A Pneumonia Enzoótica Suína (PES) tem como agente etiológico primário a bactéria Mycoplasma hyopneumoniae. O diagnóstico da PES envolve manifestações clínicas na granja, lesões macroscópicas visualizadas no frigorífico e técnicas laboratoriais, tais como isolamento por cultura, histopatologia, imunoistoquímica, ELISA e outros testes sorológicos e PCR. O objetivo deste trabalho foi estabelecer a comparação entre resultados da PCR e achados macroscópicos típicos em pulmão com PES e avaliar a eficácia dos resultados da PCR com extração por fenol-clorofórmio e kit comercial, em fragmentos de pulmão de suínos. A eficácia da PCR foi avaliada pela comparação entre o método clássico de extração de DNA por fenol-clorofórmio e por Kit comercial. Fragmentos de pulmões de 18 suínos com lesões características de PES e 17 sem lesões foram submetidos a extração de DNA e posterior PCR. As lesões pulmonares com PES, foram PCR positivas na PCR em 94.44%, contra 76.47% dos pulmões sem PES. A concordância por Kappa entre macroscopia e PCR foi 18%, não significativo, (p>0.05).

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http://dx.doi.org/10.21708/avb.2019.13.4.8532

Dos 35 fragmentos de pulmões processados por fenol-clorofórmio, 85.71% foram positivos por PCR, contra 28.57% pelo kit comercial. A concordância por Kappa entre os métodos de extração de DNA foi 57%, estatisticamente significativa (p<0,05), mas, o Odds ratio foi 15 (CI 4.52 to 49.68), o que significa dizer que a probabilidade de positividade por PCR pelo método de fenol-clorofórmio foi 15 vezes maior do que pelo kit comercial. Os resultados da macroscopia e PCR foram discordantes, sendo a extração por fenol-clorofórmio o melhor entre ambos os procedimentos usados.

INTRODUCTION

Enzootic Swine Pneumonia (PES) is a cosmopolitan respiratory disease of a chronic character, with high morbidity and low mortality, characterized by a catarrhal bronchopneumonia that manifests clinically as a chronic cough and growth retardation (CONCEIÇÃO *et al.*, 2006; HOLST *et al.*, 2015; MAES *et al.*, 2018). Its transmission occurs by direct and indirect contact with respiratory secretions and aerosols eliminated in the cough (CONCEIÇÃO *et al.*, 2006). It is a disease of great economic importance since it causes losses in zootechnical performance, with weight reduction, and makes pigs susceptible to secondary infections due to the ability of this bacterium to cause immunosuppression (ARTIUSHIN *et al.*, 1993; CONCEIÇÃO *et al.*, 2006). PES has as its primary etiologic agent the bacterium *Mycoplasma hyopneumoniae*, belonging to the genus *Mycoplasma*–of the Mollicutes class. It is an extremely small fastidious bacterium, with no cell wall, and a small genome (1140kb) of DNA (SILVA *et al.*, 2009).

The diagnosis of PES involves the association of clinical signs on farms, macroscopic lesions visualized in the slaughterhouse and laboratory techniques, such as isolation by culture, histopathology, immunohistochemistry (RIBEIRO *et al.*, 2004), Serology, ELISA (TAMIOZZO *et al.,* 2011) and PCR (ARTIUSHIN *et al.*,1993; OTAGIRI *et al.*,2005). PCR is a fast, sensitive, and specific molecular technique (SILVA *et al.*, 2009; GARZA-MORENO *et al.*, 2018).

The present study aimed to establish the comparison of PCR results with typical macroscopic findings in lungs with PES and to evaluate the efficacy of PCR results with extraction by phenol-chloroform and commercial kit, in fragments of swine lungs in the slaughterhouse.

MATERIAL AND METHODS

Seventeen samples of lung fragments of pigs (control group) without macroscopic lesion and 18 samples (case group) with macroscopic lesions obtained from three slaughterhouses under the State Inspection Service (SIS) located in the North Fluminense region of the state of Rio de Janeiro were used in this study. Macroscopic pulmonary lesions suggestive of *M. hyopneumoniae* were defined macroscopically as purple to greyish areas of pulmonary consolidation, usually located in the cranial-ventral parts of the pulmonary lobes. The lesions with hepatitis were collected with butcher in the area of inspection of the SIS taking the sterilization care at 96 º C for 5 minutes between the fragments. For the collection of clinical specimens, we used the methodology described by Overestiansky *et al.* (2005) and subsequently the samples were stored in polypropylene microtubes and refrigerated in thermal boxes for dispatch to the laboratory.

Initially, the samples were macerated in 1.0 mL of Tris-EDTA buffer (TE) pH 8.3. The standard strain of *Mycoplasma hyopneumoniae* ATCC 25934 was used as positive control. The phenol-chloroform extraction method used was an adaptation of the one cited by Sambrook *et al.* (1989). First the samples were centrifuged at 13500 rotations per minute (RPM) for 20 minutes at 10 °C and then the supernatant was discarded. The sediment was added with 400 µl of TE dextrose, 100 µL of proteinase K (240 M g/mL), and 30 ml of dodecyl sodium sulfate (SDS) 10% and incubated in a thermal block for 30 minutes followed by an ice bath for five minutes. After this step, two extraction stages with phenol were performed. A volume of 500 µL of buffered phenol was added to the samples and homogenized gently by inversion for 10 minutes and then centrifuged at 13500 rpm for 30 minutes at 55 °C. To perform the second washing with phenol, the supernatant was transferred to a new microtube; the same procedure was repeated, centrifuging only for five minutes. Then, the supernatant was transferred to a new microtube, and 500µL of chloroform was added. The samples were homogenized for three minutes by inversion and centrifuged at 13500 rpm for five minutes at 10º C. The supernatant was transferred to a new microtube and 1000 µl of absolute ethanol was added. This material was stored at -20 °C overnight. After this period, the samples were centrifuged at 13500 rpm for 20 minutes at 10º C and the alcohol was discarded and dried at room temperature. After complete drying, the sample was resuspended with 200 µL of TE and stored at -20 °C. The other extraction method used the kit Promega Wizard-20 °C® SV genomic DNA Purification System (Promega, Madison, U.S. a.), following the guidance of the manufacturer.

Primers were used based on a previously described DNA sequence (ARTIUSHIN *et al.*, 1993). Each reaction consisted of 1 U of Taq DNA polymerase, 0.2 µM of each DNTP, 16 pmol of each primer, 50 mM of buffer 10x [Tris-HCL 20mm (PH 8.4), KCL 50 MM], MgCl2 4mM. The result was observed in agarose gel 1.5% stained with ethidium bromide and visualized in violet transiluminator after electrophoretic run.

To infer the correlation between the sample positivity and the extraction method used, the Kappa agreement test was performed using the Bioestat 5.0 software.

RESULTS AND DISCUSSION

Lung fragments of 35 pigs were collected in the slaughterhouse. Of these, 18 presented macroscopic lesion of PES in the lung and 17 did not. From the lungs of pigs that presented lesions for PES in the macroscopic examination, 94.44% (17/18) were positive by PCR and 5.56% (1/18) negative, while the samples diagnosed as negative for macroscopic examination, 76.47% (13/17) were positive and 23.53% (4/17) negative in PCR (table 1). The *Kappa* index obtained between the macroscopic examination and PCR was 0.18 (18%), that is, these methods were little concordant, with no association between them $p > 0.05$. Similar results were obtained by Teixeira *et al.* (2003) when they reported that the macroscopic lesions in the lungs were not sensitive to detect PES, since some samples that presented no macroscopic injuries to PES obtained positive results in PCR, as well as the lung samples with lesions were negative in PCR. Some samples were positive for PCR and presented no pulmonary lesions possibly due to the natural healing process, by which the microorganism remains in the respiratory tract, transforming the animal into an asymptomatic pathogen carrier (PULGARÓN *et al.*, 2015; SIQUEIRA *et al.*, 2017). Moreover, the detection of *M. hyopneumoniae* in the lungs without macroscopic lesions of the type pneumonia may also be related to the infectious process, and in these cases, the use of PCR capable of detecting small amounts of microorganisms per sample , which would be considered false negatives by less sensitive methods. The presence of samples with lesions, but negative for PCR, probably occurred due to infection by other respiratory agents, such as *Actinobacillus pleuropneumoniae* and *Mannheimia haemolytica*. These microorganisms are causative agents of respitatory disease in pigs and may cause similar lesions caused by *M. hyopneumoniae* (SASSU *et al.*, 2017).

Li *et al.* (2019) observed a relationship between the presence of lesions and positivity in PCR. On the other hand, Ribeiro *et al.* (2004) observed a positive correlation between macroscopic and microscopic diagnoses and between histological and immunohistochemical techniques. However, these authors observed a low correlation between the macroscopic and immunohistochemical methods, being the immunohistochemistry of high sensitivity and moderate specificity.

Table 1. Relationship between PES and detection of *Mycopasma hyopneumoniae* by PCR in lung fragments*.

	With macroscopic PES lesions	Without macroscopic PES lesions	Total
Positive in PCR	17 (94.44%)	13 (76.47%)	30
Negative in PCR	1 (5.56%)	4 (23.53%)	5
	18	17	35

* Kappa concordance = 0.18; expected Concordance = 0.60; $p > 0.05$.

Of the 35 lung fragments processed by phenol-chloroform, 85.71% (30/35) were positive and 14.29% (5/35) negative in the PCR, while by the commercial Kit, 28.57% (10/35) were positive and 71.43% (25/35) negative (table 2). The Kappa agreement (0.18) between the methods of DNA extraction was regular and the odds ratio was equal to 15 (IC 4.52 a 49,68). Consequently, the probability of obtaining positive PCR was 15 times greater with 4.52 to phenol-chloroform than the extraction by the commercial Kit.

Table 2. Relationship between phenol-chloroform and commercial Kit in the DNA extraction of *M. hyopneumoniae* by PCR*

	Phenol-chloroform	Commercial kit
Positive	30	10
Negative	5	25
Total	35	35

* * Kappa agreement = 0.57; observed agreement = 0.79; $p < 0.05$. Odds ratio = 15; IC 4.52 to 49.58.

CONCLUSIONS

The macroscopic findings of swine lungs in the slaughterhouse under SIE showed no agreement with their results obtained by PCR in the diagnosis of PES. Extraction by the phenol-chloroform technique from lung tissue samples proved to be more effective than that by the commercial kit; however, eventually for other types of samples this type of extraction may not be the best alternative.

ACKNOWLEDGEMENTS

The authors thank CNPq and FAPERJ for the financial support for the execution of this research.

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