

Detection of *Mycoplasma hyopneumoniae* in lungs and nasal swabs of pigs by nested PCR

[*Detecção de Mycoplasma hyopneumoniae em pulmões e suabes nasais de suínos por nested PCR*]

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ABSTRACT

Fifty-four samples were collected from growing and finishing pigs for the molecular diagnosis of enzootic porcine pneumonia. Nineteen lung fragments were obtained from pigs that showed signs of respiratory disease and 35 nasal swabs were obtained from clinically healthy pigs. For the detection of the bacterial genome in the samples, the nested PCR technique was used to amplify a fragment of 706bp. This fragment was subsequently cloned and sequenced. The sequence of obtained nucleotides was compared with six other sequences of *Mycoplasma hyopneumoniae* and 11 sequences of other bacteria available in the Genbank. To measure the sensitivity of the nested PCR, serial dilutions (10^{-1} to 10^{-15}) of cloned fragments were conducted based on the concentration of 300ng. Ten lung fragments and eight nasal swabs showed positive for *M. hyopneumoniae* and the limit of detection was estimated to be 0.3fg DNA cloned. The sequence of nucleotides obtained showed 99.1% homology with the other sequences of *M. hyopneumoniae*, demonstrating that the nested PCR used in this study may provide an important diagnostic tool for the detection of this agent.

Keywords: swine, enzootic pneumonia, *Mycoplasma hyopneumoniae*, nested PCR

RESUMO

Foram coletadas 54 amostras de animais em fase de crescimento e terminação para o diagnóstico molecular da pneumonia enzoótica suína. Dezenove fragmentos de pulmão foram obtidos de suínos que apresentavam sinais de doença respiratória e 35 suabes nasais foram obtidas de suínos clinicamente saudáveis. Para a detecção do genoma bacteriano nas amostras, foi utilizada a técnica de nested PCR que originou um fragmento de 706pb, o qual foi, posteriormente, clonado e sequenciado. A sequência de nucleotídeos obtida foi comparada com outras seis sequências de *Mycoplasma hyopneumoniae* e 11 sequências de outras bactérias disponíveis no Genbank. Para medir a sensibilidade da nested PCR, foram realizadas diluições seriadas (10^{-1} a 10^{-15}) do fragmento clonado, partindo da concentração de 300ng. Dez fragmentos de pulmões e oito suabes nasais apresentaram resultado positivo para *M. hyopneumoniae* e o limite de detecção foi estimado em 0,3fg de DNA clonado. A sequência de nucleotídeos obtida foi de 99.1% de homologia com as outras sequências de *M. hyopneumoniae*, demonstrando que a nested PCR utilizada neste estudo pode ser uma importante ferramenta de diagnóstico para a detecção desse agente.

Palavras-chave: suíno, pneumonia enzoótica, *Mycoplasma hyopneumoniae*, nested PCR

Recebido em 27 de maio de 2008

Aceito em 15 de outubro de 2008

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INTRODUCTION

Mycoplasma hyopneumoniae is a member of *Mollicutes* class presenting a small double stranded circular DNA genome of 1,140kb (Minion et al., 2004). Enzootic porcine pneumonia (EPP), also called mycoplasmal pneumonia, presents *M. hyopneumoniae* as the primary agent (Hege et al., 2002). EPP is a chronic respiratory disease that causes major economic losses to the pig industry (Thacker, 2006). This disease is found in almost all productive areas in Brazil (Sobestiansky et al., 2001). Infection occurs during direct contact with respiratory secretions from carrier animals, and also by airborne transmission (Stark et al., 1998).

Micoplasmal infection causes pneumonia characterized by a sporadic, dry and non-productive cough, retarded growth rate, and inefficient utilization of feed (Ross, 1999). Secondary infections caused by bacterial pathogens such as *Actinobacillus pleuropneumoniae* (Yagihashi et al., 1984) and *Pasteurella multocida* (Amass et al., 1994) can aggravate clinical manifestations in pigs that are primarily infected with *M. hyopneumoniae*. *M. hyopneumoniae* rapidly spreads under favorable environmental conditions in growing and finishing pigs (Ribeiro et al., 2004). The disease is traditionally controlled by antibiotics but the role of management and housing conditions in EPP development have shown to be very effective (Maes et al., 2000).

Several methods for *M. hyopneumoniae* detection have been developed, and an accurate diagnosis of EPP is a prerequisite for combating the disease (Mayor et al., 2008). The isolation of *M. hyopneumoniae* by culture has not been currently performed by diagnostic laboratories mainly due to the fact that this mycoplasma is one of the most fastidious slow growing microorganism (Marois et al., 2007). Serological analysis specificity using polyclonal antibodies is ambiguous, since cross-reactions occur with two other porcine mycoplasmas such as *M. flocculare* and *M. hyorhinae* (Freeman et al., 1984; Strasser et al., 1992). Techniques derived from molecular biology to detect mycoplasmas are being applied in veterinary medicine, since the polymerase chain reaction test (PCR) has been widespread used in early diagnosis of diseases (Buzinhan et

al., 2007). PCR technology is ideally recommended for *M. hyopneumoniae* diagnosis because it is fast, specific, and can be performed on both living and dead animals (Calsamiglia et al., 2000). Several PCR assays have been developed to specific *M. hyopneumoniae* DNA fragments (Verdin et al., 2000; Kurth et al., 2002). At present, the diagnosis of mycoplasmal pneumonia is based on clinical signs and histopathological lesions in many diagnostic laboratories (Whitford et al., 1994). It is important to notice that the presence of *M. hyopneumoniae* is evidenced during the whole disease course while clinical and pathological signs are dependent on the disease stage (Sorensen et al., 1997; Calsamiglia et al., 2000).

The objective of the present study was to evaluate a nested PCR assay and its sensitivity for the detection of *M. hyopneumoniae* from nasal swabs and lung samples as a diagnostic tool of enzootic pneumonia in pigs.

MATERIAL AND METHODS

A total of 54 samples of nasal swabs and lung fragments from different pigs were used. Nineteen lung fragments were obtained from both growing and finishing pigs that presented signs of respiratory disease. Thirty-five samples of nasal swabs were randomly obtained from both growing and finishing animals aiming to evaluate either the presence or absence of *M. hyopneumoniae* in clinically healthy animals. Such animals were supervised until they were slaughtered and did not show any kind of clinical signs of the studied disease. Samples randomly obtained were suspended in 2mL of 0.1M phosphate buffered saline solution (PBS) and frozen at -20°C.

Samples of nasal swab were collected in farms in Rio de Janeiro and at the Universidade Federal de Viçosa. Lung fragments were donated from a Veterinary Microbiology Company.

A phenol-chloroform extraction method was used to extract DNA from lung samples as described by Sambrook et al. (1989). Nasal swab samples were centrifuged at 12,000g for 30min, and then the cell pellets were resuspended in 1mL of lysis buffer [Tris-HCl 10mM (pH 7.5), MgCl₂ 5mM, NaCl 10mM] and the DNA was

extracted using phenol-chloroform (Sambrook et al., 1989).

PCR reactions were carried out using two pairs of primers based on a *M. hyopneumoniae* DNA probe sequence (I141-accession number U 02537), previously described by Blanchard et al. (1996a). These primers amplified a part of the multidrug resistance protein gene, which is a member of *ATP-binding cassette transporter genes* (Higgins, 1992). Amplifications were performed in a final volume of 25mL. The reaction mixture consisted of 1.0U of *Taq* DNA polymerase¹, 200µM of each dNTP, 0.5µM of each primer, 10X assay buffer [Tris-HCL 20mM (pH 8.4), KCl 50mM], and MgCl₂ 2mM. The primers used in the PCR were Hp1 (5'-TTCAAATTATAACCTCGGTC-3') and Hp3 (5'-AGCAAATTTAGTCTCTCTGTC-3'), and around 200ng of purified DNA were used as a template. The amplification of DNA was achieved by 40 cycles of 95°C for 1min, 52°C for 1min, and 72°C for 2min. The combination of Hp4 (5'-CGCTTTAGTACCGATATGGG-3') and Hp6 (5'-GCCATTTCGCTTATATGGTGA-3') was used for the nested PCR. Two microlitres of the PCR product were transferred to the nested PCR reaction. The reaction mixture was amplified for 40 cycles at 95°C for 1min, 55°C for 1min, and 72°C for 1min. In each PCR reaction, a negative control consisting of swine genomic DNA free of *M. hyopneumoniae*, as well as a DNA positive control extracted from a pure culture of *M. hyopneumoniae*, were included. PCR products were analyzed with 1% agarose gel in a TBE buffer (89mM Tris-HCL, 89mM borate, and 2mM EDTA) stained with ethidium bromide and visualized under UV illumination.

Nested PCR products were purified by GFX™ PCR DNA and a gel band purification kit² and cloned into TOPO TA Cloning®³. *Escherichia coli* DH5α competent cells were used for transformation. Recombinant plasmids were extracted as described by Sambrook et al. (1989). Direct nucleotide sequencing of both strands was performed on an ABI Prism 377 Genetic Analyzer⁴ with a commercially available kit⁵ according to the protocol of the manufacturer using M13 primers from TOPO TA Cloning® kit.

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⁵ABI PRISM BigDye III Terminator Cycle Sequencing Ready Reaction®, Applied Biosystems, Foster City – USA.

A nucleotide sequence consensus was generated by the sequencing of three different recombinant clones from the same isolate which was later deposited in the GenBank under the accession number DQ364651. Nucleotide sequences were searched for with homologies in the GenBank by the BLAST program (Altschul et al., 1990) provided by NCBI, USA. Nucleotide sequences retrieved from GenBank (Table 1) were aligned using the Clustal W version 1.8 multiple sequence alignment program (Thompson et al., 1994).

Table 1. Bacteria sequences used in nucleotide analysis retrieved from GenBank

GenBank accession No.	Species	Location
AE017334	<i>Bacillus anthracis</i>	USA
AE017038	<i>Bacillus anthracis</i>	USA
AE017012	<i>Bacillus cereus</i>	France
CP000001	<i>Bacillus cereus</i>	USA
AE017355	<i>Bacillus thuringiensis</i>	USA
AE015940	<i>Clostridium tetani</i>	USA
AE017146	<i>Helicobacter hepaticus</i>	USA
AE000615	<i>Helicobacter pylori</i>	USA
AE017244	<i>Mycoplasma hyopneumoniae</i>	Brazil
AY143369	<i>Mycoplasma hyopneumoniae</i>	Switzerland
AE017332	<i>Mycoplasma hyopneumoniae</i>	USA
NC_006360	<i>Mycoplasma hyopneumoniae</i>	USA
U02537	<i>Mycoplasma hyopneumoniae</i>	France
U19289	<i>Mycoplasma hyopneumoniae</i>	France
BA000017	<i>Staphylococcus aureus</i>	Japan
AE016750	<i>Staphylococcus epidermidis</i>	China
NC_003143	<i>Yersinia pestis</i>	USA
DQ364651	<i>Mycoplasma hyopneumoniae</i>	Brazil

RESULTS AND DISCUSSION

PCR reactions performed with Hp1 and Hp3 primers yielded a detectable DNA fragment of 1561bp. A visible band of the expected size was revealed on gel (data not shown). The nested PCR performed with Hp4 and Hp6 primers amplified a 706bp fragment, as shown in Fig. 1.

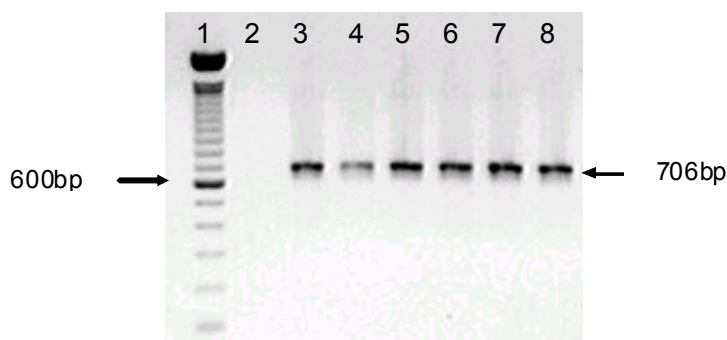


Figure 1. Nested PCR products obtained from the lungs and nasal swabs of pigs. Lane 1: molecular size standard (100bp ladder; Gibco BRL Life Technologies). Lane 2: Negative control: swine genomic DNA free of *Mycoplasma hyopneumoniae*. Lane 3: positive control: *M. hyopneumoniae* genomic DNA. Lanes 4 and 5: Nested PCR products from nasal swabs. Lanes 6 to 8: nested PCR products from lungs. The position of the specific fragment is indicated (706bp).

Fifty-four samples consisting of 19 lung fragments and 35 nasal swabs were analyzed in order to standardize an EPP molecular diagnostic technique. Ten lung fragments and eight nasal swabs presented positive results to *M. hyopneumoniae*.

Positive results obtained with nasal swabs will be of great importance to clinically healthy animals, supervising the quiet dispersal of the infectious agent inside the farm and making *in vivo* diagnostic of enzootic pneumonia possible. The fact of few nasal swab samples presenting positive results may be due to either absence of clinical signs on tested animals or the absence of the microorganism. Ross (1992) described that diagnosis done by *M. hyopneumoniae* cultivation from nose samples is extremely difficult. That issue could be amended by using the nested PCR technique, once the results found in this work demonstrated the detection of this agent in samples obtained from nasal swabs. Marois et al. (2007) showed that tracheal swabs and tracheobronchiolar washings were the most effective samples to detect *M. hyopneumoniae* compared to nasal or tonsillar swabs, however, tracheal swabs are more difficult to perform under field conditions with older pigs.

According to Calsamiglia et al. (1999), the lung samples stood out as the most appropriate source of *M. hyopneumoniae* detection once this microorganism is known to be present in the

nostrils in much smaller numbers than in the lower airways. Verdin et al. (2000) demonstrated that *M. hyopneumoniae* was probably confined to the lower respiratory tract in six-month-old pigs. Otagiri et al. (2005) also found that lung sample detections were higher than in nasal swabs from the same animals.

Once Mycoplasma is apparently present in small quantities in nasal swabs than in lower respiratory tracts, the sensitiveness of a diagnosis method is really important. The sensitivity of nested PCR was also evaluated in this study with 10-fold dilutions of *M. hyopneumoniae* cloned DNA (Fig. 2). A DNA fragment of 706bp was successfully amplified showing as little as 0.3fg of cloned DNA, a more accurate result than that previously obtained by Verdin et al. (2000), who conducted nested PCR experiments with 10-fold dilutions of *M. hyopneumoniae* genomic DNA and obtained 1fg of genomic DNA. Calsamiglia et al. (1999) described a nested PCR analysis using specific primers for *M. hyopneumoniae* 16S ribosomal DNA. The sensitivity of this nested PCR was approximately 100fg of genomic DNA.

The specificity of these primers for *M. hyopneumoniae* had been evaluated before by Blanchard et al. (1996a), when DNA from different porcine infectious bacteria (including other Mycoplasma species) had not yielded any amplified product.



Figure 2. Sensitivity of the nested PCR assay of *M. hyopneumoniae* cloned fragment. Lane 1 contained the molecular size standards (100bp ladder; Gibco BRL Life Technologies). Lanes 2 and 3 contained negative and positive controls, respectively. The amount of DNA added to each reaction shown in lanes 4-18 was 300ng, 30ng, 3ng, 300pg, 30pg, 3pg, 300fg, 30fg, 3fg, 0.3fg, 30ag, 3ag, 0.3ag, 30zg, and 3zg. The position of the specific fragment is indicated (706bp).

The nucleotide sequence of the nested PCR product cloned in TOPO TA Cloning[®] showed to be a part of multidrug resistance protein gene. The product of this gene is involved in the transport of functions resembling those of the eukaryotic multidrug resistance (MDR) protein family. Such a product might be essential for *M. hyopneumoniae* pathogenicity once it is involved in drug resistance (Blanchard et al., 1996b).

Nucleotide analysis showed that the DQ364651 isolate found in this study had a higher relation to other *M. hyopneumoniae* sequences from Brazil and the United States, 98.9% identity, and less

homology to French sequences, 97.9% identity. The obtained sequence was compared to other bacterial multidrug resistance protein genes, being more closely related to *Clostridium tetani*, *Helicobacter pylori*, and *Helicobacter hepaticus*, 43.7%; 40.3%; and 43.0% identity, respectively. The other bacterial sequences retrieved from GenBank exhibited extremely low identity (24-36%) to the *Mycoplasma* isolated in this study (Fig. 3). Once this sequence presented low identity to other bacterial species and high identity between *M. hyopneumoniae* sequences this nested PCR assay represents an interesting tool for molecular diagnosis of *M. hyopneumoniae*.

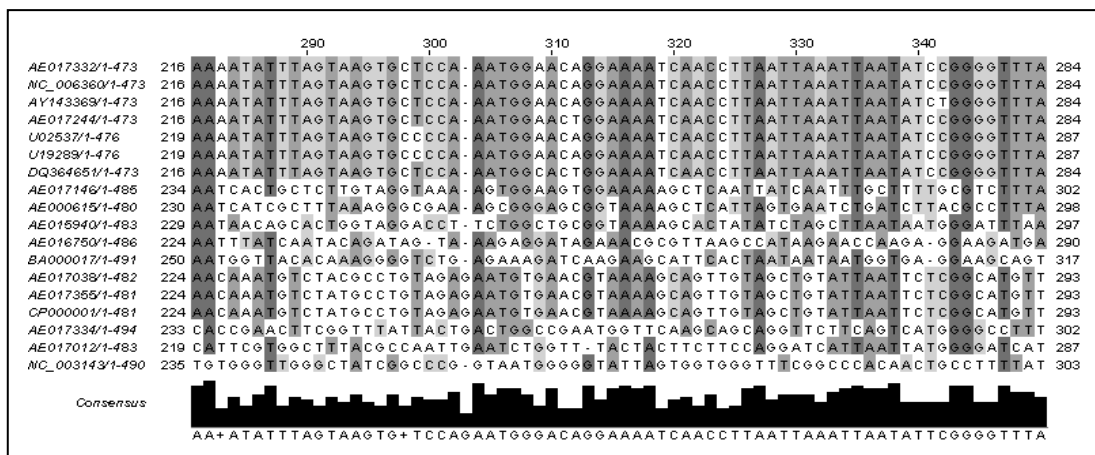


Figure 3. Partial multiple nucleotide alignment of nucleotide sequence of multidrug resistance protein gene, obtained by ClustalW (1.8). The regions with conserved nucleotides are shown in three levels according to the identity. Nucleotide sequence of the isolate of this study is referred to as DQ364651.

⁶Invitrogen - San Diego, USA.

The usefulness of a nested PCR assay for its fast and sensitive detection of *M. hyopneumoniae* from lung fragments and nasal swabs has been demonstrated in this report. Once the lung fragment detection is performed on dead animals, nasal swabs are more suitable for *M. hyopneumoniae* detection as it is performed on living animals. Additionally, sample collections are easier, and it could be used to monitor the disease status in pig herds. The isolate fragment found by this work displayed a 99.1% overall nucleotide homology with *M. hyopneumoniae* sequences retrieved from the GenBank demonstrating that the nested PCR target is highly preserved between *M. hyopneumoniae* which supports the fact that the PCR used in this study would provide an accurate diagnosis.

ACKNOWLEDGMENTS

We are thankful to Dr. Jalusa Deon Kich from Embrapa Suínos e Aves, who kindly provided the positive control of PCR reactions. We also thank MICROVET for providing the swine tested samples.

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