

DETECTION OF *MYCOPLASMA HYOPNEUMONIAE* BY POLYMERASE CHAIN REACTION IN SWINE PRESENTING RESPIRATORY PROBLEMS

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ABSTRACT

Since *Mycoplasma hyopneumoniae* isolation in appropriate media is a difficult task and impractical for daily routine diagnostics, Nested-PCR (N-PCR) techniques are currently used to improve the direct diagnostic sensitivity of Swine Enzootic Pneumonia. In a first experiment, this paper describes a N-PCR technique optimization based on three variables: different sampling sites, sample transport media, and DNA extraction methods, using eight pigs. Based on the optimization results, a second experiment was conducted for testing validity using 40 animals. In conclusion, the obtained results of the N-PCR optimization and validation allow us to recommend this test as a routine monitoring diagnostic method for *Mycoplasma hyopneumoniae* infection in swine herds.

Key-words: Swine Enzootic Pneumonia, *Mycoplasma hyopneumoniae*, diagnosis, Nested- PCR.

INTRODUÇÃO

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) is the primary etiological agent of the Swine Enzootic Pneumonia (SEP), that leads to a non-productive cough, mild fever, weight gain loss, high morbidity, and low mortality (12,17). SEP is considered one of the most worldwide common respiratory diseases and is responsible for high economic loss due to secondary infections. Its diagnosis can be performed by anamnesis, clinical signs described above, and gross lesions detected at slaughterhouses such as hepatized lung areas. However, since several microorganisms may cause similar alterations, it is not possible to state that *M. hyopneumoniae* is the causative agent of such lesions and clinical signs (8,12,23,27).

Although definitive PES diagnostic can be obtained by microorganism isolation, this technique is laborious and requires at least 15 days for colony growth. These features make isolation procedures impractical for daily routine diagnosis. Antibody identification by ELISA or IFI is a diagnostic alternative, but it may show non-specific reactions with other porcine mycoplasmas, such as *M. hyorhinae* and *M. flocculare* (15,19,23).

Nowadays, fastidious microorganisms have been detected by molecular biology techniques, such as DNA or RNA hybridization and Polymerase Chain Reaction (PCR). These techniques are faster and more specific for microorganism detection (6); and since mycoplasmas isolation is impractical in daily routine of several laboratories, PCR assay can improve the sensitivity of their direct diagnostic. Accordingly, this technique may be feasible to apply for detection of *M. hyopneumoniae* from pig clinical samples such as tracheal washings nasal swabs, and lung fragments (2,7,13,18,28).

The Nested-PCR (N-PCR) is a variation of the standard PCR that involves second amplification of the PCR product, using internal specific primers complementary to the first amplification nucleotide sequence (3). According to TANG *et al.* (1997), this second amplification increases the PCR sensitivity because of the double amplification process, and also verifies the first reaction specificity.

Thus, this paper proposes the optimization of a N-PCR technique for the detection of 16S rRNA gene fragments of *M. hyopneumoniae* through the evaluation of different sampling sites, sample transport media, and DNA extraction methods.

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MATERIAL AND METHODS

In a first experiment, eight pigs at 10 to 12 weeks old from Unidade Demonstrativa de Suínos do CNPSA- EMBRAPA were selected. They showed SEP clinical signs, no mycoplasma vaccination history, and were positive by ELISA (CNPSA-EMBRAPA) for *M. hyopneumoniae* infection. These animals were used to analyze two biological samples, nasal and tonsil swabs; two sample transport media; and five DNA extraction methods.

Initially, 10 mL of blood were collected from each animal by venipuncture of the anterior cava vein using disposable syringe and 80 X 10 needles. After clot formation, blood samples were centrifuged for five minutes at 3000 x g. The obtained serum was stored at -20°C until ELISA procedures.

A total of 136 swabs (tonsil and nasal) were collected from the eight animals in a period of 14 days with a 4 day interval. The swabs were stored in two sample transport media, 68 in FRIIS media (34 tonsil swabs and 34 nasal swabs) (10) and 68 in saline (NaCl 0.85%) (34 tonsil swabs and 34 nasal swabs). All samples were stored in microtubes with 0.5 mL of media, inside a box containing ice, and sent to the laboratory. Then, the samples were vortex mixed and 200 µl were used for DNA extraction. For N-PCR optimization, five DNA extraction methods were evaluated: A) silica/guanidinium thiocyanate (5); B) boiling/ice (9); C) triton X 100/ proteinase K (21); D) phenol/chloroform (7) E) phenol/chloroform / isoamyl alcohol (22). A total of 50 µl of the extracted DNA was stored in microtubes at -20°C until PCR assays.

In a second experiment, 40 pigs of 10 to 12 weeks old presenting coughing and sneezing were used. They were from a farm with a history of lung lesions at the slaughterhouse (hepatized areas) and positive results for *M. hyopneumoniae* serology by ELISA (CNPSA-EMBRAPA).

Blood samples for ELISA were also collected as described in the first experiment. The swabs were obtained from a single visit to the farm. Two tonsil and two nasal swabs were collected from each animal for a total of 160 swabs. From the 80 nasal swabs, 40 swabs were stored in saline and 40 in FRIIS media. The same procedures were adopted for the 80 tonsil swabs. Sample conservation and transportation were followed as described in the first experiment.

Based on the results of the first experiment, the silica/guanidinium thiocyanate method (Boom) was used for the tonsil and nasal swabs stored in saline and, the boiling/ice method (Fan) was used for the tonsil and nasal swabs stored in FRIIS media.

The specie-specific primers utilized in this study were complementary to conserved mycoplasma sequences of the 16S rRNA gene. The external primers Mh-up and Mh-do (PCR), described by MATSSON *et al.* (1995), and the internal primers NMh-up and NMh-do (N-PCR), described by CALSAMIGLIA

et al. (1999) are presented in Table 1. All primers were synthesized by Invitrogen® do Brasil.

During the PCR optimization, different annealing temperatures for external and internal primers, and primers and MgCl₂ concentrations were analyzed in independent reactions. These parameters were analyzed keeping Taq DNA polymerase, buffer PCR and dNTPs in constant concentrations.

For the PCR assay, 2 µl of DNA sample were added in a 20 µl PCR-MIX containing one unit of Taq DNA polymerase, 1X PCR buffer (20 mM Tris HCl pH 8.4 and 50 mM KCl), and 1 mM of dNTP's. Primers and MgCl₂ concentrations varied according to the type of the primer. For the N-PCR assay, the volume of the product of the first amplification and N-PCR MIX were 1 µl and 20 µl, respectively. MgCl₂ concentrations of 2, 2.5, 3, and 3.5 mM for internal and external primers were tested in separate reactions. Different primer concentrations were also evaluated (2, 5, 10, 15, 20, 25, and 30 pmol). For evaluation of the annealing temperature, a gradient of temperature was used with thermal amplitude of 7°C around the average temperature of 52°C.

Amplification processes were taken in a thermocycler (Mastercycler gradient 5331 – Eppendorf) according to the following conditions: 94°C for 4 minutes; 35 cycles at 95°C for 45 seconds, gradient of temperature for 1 minute, 72°C for 2 minutes; followed by a final extension at 72°C for 5 minutes. For each amplification, negative (ultrapure water) and positive (*M. hyopneumoniae* - ATCC 25934) controls were added.

A total of 10 µl of each PCR product and a 100 bp molecular weight (Amersham Pharmacia Biotech®) were separated by electrophoresis in a 2% agarose gel containing ethidium bromide, 0.5 µg/ml in TEB 1X buffer (Tris 0.1 M; 0.5 M EDTA pH 8.0; boric acid, pH 6.0), under constant voltage of 100V for 50 minutes. The gels were viewed and photographed under UV light using a Vilber Lourmat imaging system.

The reference strain *M. hyopneumoniae* (ATCC 25934) was used for the sensitivity test (*M. hyopneumoniae* detection limit). It was cultured in FRIIS media at 37°C for 10 days and diluted in the same media until 10⁻⁸. A total of 100 µl of each dilution was plated in FRIIS media plates and incubated at 37°C in microaerophilia. After 10 days of incubation, Colony Forming Units (CFU) were counted for DNA extraction and PCR and N-PCR assays.

The spectrophotometer was used to determine the sensitivity using 1 mL of the *M. hyopneumoniae* initial culture. DNA extraction was performed by Boom method and the DNA was quantified in µg/µl.

N-PCR specificity was evaluated using commonly found microorganisms of the swine respiratory tract: *M. hyopneumoniae* (ATCC 25934), *M. hyorhinis* (ATCC 17981), *M. hyosynoviae* (ATCC 25591), *M. flocculare* (MF) (ATCC 27716), *Actinobacillus pleuropneumoniae* serotype 5B cepa L20, *Haemophilus parasuis* S1 (Nr4p₂), *Haemophilus parasuis* S2 (SW140 p₂), *Pasteurella multocida*, and *Bordetella*

Table 1. Primers used for fragment amplification of the 16S rRNA gene of *Mycoplasma hyopneumoniae* by Nested-PCR.

External primers	Sequence	Position	Fragment size
<i>Mh up</i> (forward)	5'GAG CCT TCA AGC TTC ACCAAGA 3'	212-233	649 bp
<i>Mh do</i> (reverse)	5'TGT GTT AGT GAC TTT TGC CAC C 3'	839-860	
Internal primers			
<i>NMh up</i> (forward)	5'ACT AGA TAG GAAATG CTC TAG T 3'	463-484	352 bp
<i>NMh do</i> (reverse)	5'GTG GAC TAC CAG GGT ATCT 3'	797-815	

bronchiseptica, *Pasteurella* and *Bordetella* strains were field isolates of Embrapa Suínos e Aves.

The DNA amplified segment was sequenced using KIT "Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit – Applied Biosystems do Brasil Ltda". The N-PCR product was precipitated using 3M sodium acetate and isopropanol for 12 hours, centrifuged at 21000 X g for 10 minutes. Then supernatant was discarded, and the sediment was suspended in TE buffer (10 mM Tris-HCl pH 7.5, and 1 mM EDTA). After precipitation, the DNA was quantified by comparison to the Lambda DNA EcoRI Hind III digest (SIGMA®) molecular weight in 0.8% agarose gel.

A total of 20 ng of the DNA sample, 5 pmol of primer NMh-up, 8 µl of the kit Big Dye, and 12 µl of ultrapure water was used for the sequencing reaction. The program profile was: 25 cycles at 96°C for 30 seconds, 50°C for 15 seconds, 60°C for 4 minutes, followed by a final step at 4°C for 15 minutes. After sequencing reaction, the product was precipitated using isopropanol and 75% ethanol. The precipitate was dried and suspended in 6¼l of "Loading buffer" and the interpretation performed in a ABI PRISMA 377 DNA SEQUENCER (Elmer Perking) system. The sequence alignment was done using a BLAST program (2006).

The N-PCR results and variables (sampling sites, sample transport media, and DNA extraction methods) were evaluated using χ^2 test by CATMOD models. The statistical software SAS (2001) was used.

RESULTS AND DISCUSSION

All the MgCl₂ concentrations resulted in predicted N-PCR products. Accordingly, the concentrations of 2.0 mM for the PCR and 2.5 mM for the N-PCR were adopted. The best annealing temperatures, without inespecific bands and with appropriate amplifications, were 65.5°C and 59.5°C for PCR and N-PCR assays, respectively.

External and internal primer concentrations evaluation did not show visual differences from 10 to 20 pmol. Thus, the chosen concentration was 10 pmol for the PCR and N-PCR.

Five DNA extraction methods, two biological samples, and two sample transport media were evaluated in the first experiment. Different DNA extraction methods have been

described in the literature with a high variability in sampling sites and sample conservation for N-PCR optimization (2,3,7,25). In the present study, DNA extraction by Boom method showed better results for nasal and tonsil swabs stored in saline; and the Fan method showed better results for nasal and tonsil swabs stored in FRIIS media (Table 2).

In the second experiment, N-PCR results were analyzed using 160 swabs from 40 animals and the following variables: sampling sites, sample transport media, and DNA extraction methods (Table 3). Although 38 (95.0%) out of the 40 tonsil samples in saline presented positive results in N-PCR and only 32 (80.0%) of the nasal samples in saline showed positive results, these results were not statistically different ($p < 5\%$) using the Boom DNA extraction method. However, using the Fan technique, there was a statistical difference ($p < 5\%$) between the 29 (72.5%) positive tonsil swabs and 06 (15.0%) positive nasal swabs. Although statistical difference could not be observed between Fan method using nasal swabs in saline and Boom using tonsil swabs in FRIIS media, Boom technique presented better results than Fan ($p < 5\%$) because of higher detection of positive results.

Comparing tonsil and nasal swabs, the number of positive samples was higher using tonsil swabs. This fact may be explained by possible blood contamination due to nasal lesions

Table 2. Number of positive samples of N-PCR collected from 8 finishing pigs using five DNA extraction methods, two types of sample transport media, and two types of swabs.

Media	FRIIS		Saline	
	Tonsil	Nasal	Tonsil	Nasal
Swab				
Extraction method				
BOOM	3	4	6	7
FAN	7	3	5	2
CBIOT	6	3	3	4
CALSAMIGLIA**	2	1	3	2
SAMBROOK**	2	0	1	0
Total	20	11	18	15

*T: Tonsil; N: Nasal; **only five pigs were used.

during the sampling and/or existing lesions caused by powder, microorganisms and other factors. This contamination may lead to reaction inhibition and/or increase in DNA from other sources than mycoplasma (24). Tonsil swab reduces the occurrence of lesions during the sampling and decreases the number of false negatives. BACCARO *et al.*, (1999) have also observed a higher number of *M. hyopneumoniae* positive animals using tonsil swab when compared to nasal sample.

Several studies have suggested different sampling sites using N-PCR, such as lung fragments, tracheobronchial washings, and nasal swabs (3,7,16). In the present study, tonsil swab was a feasible sampling method for pigs, without the need of anesthesia or *post mortem* sampling.

Statistical analyses of DNA extraction methods presented in Table 3 show the superiority of the Boom technique when compared to Fan, independently of the sampling site.

M. hyopneumoniae viability in swabs stored in saline was greater when compared to swabs stored in FRIIS media (Table 3). FRIIS media was used in the present study due to recommendations of several authors for *M. hyopneumoniae* isolation (10,11,14,18). BAUSMEISTER *et al.* (1998) have found low sensitivity of the PCR using bronchoalveolar samples cultivated in FRIIS media, probably because of media component interference on PCR, especially the serum.

The DNA detection limit of the N-PCR using a spectrophotometer was 8×10^{-8} µg/µl (dilution 10^{-5} , Fig. 1), or 80 femtograms (fg) of DNA, corresponding to 80 microorganisms. Femtograms determination was reported by VERDIN *et al.* (2000), in which, 1 fg was equivalent to one microorganism, according to the mean genome size of mycoplasmas. CALSAMIGLIA *et al.* (1999) used the same primers of the present study and also found 80fg sensitivity.

Sensitivity according to CFU counts was approximately 0.9 CFU/µl. According to (20), one CFU ranged from 100 to 1000 microorganisms. The N-PCR sensibility evaluation by spectrophotometer is more accurate than colony counting, since microorganisms equivalence is close to the true microorganism number than the value obtained by CFU.

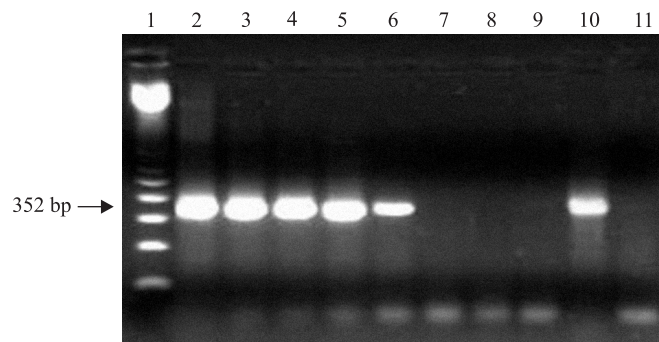


Figure 1. Sensitivity test results of N-PCR technique used for DNA detection of *M. hyopneumoniae* 16SrRNA gene: 1- 100bp ladder (Amersham pharmacia biotech); from 2 to 9- DNA dilutions (Boom DNA extraction method) in ultrapure water from 10^{-1} to 10^{-8} ; 10- Positive control (*M. hyopneumoniae* – ATCC 25934); 11- N-PCR negative control.

This fact is reported by several authors that determine N-PCR sensitivity, such as VERDIM *et al.* (2000) with a sensitivity of 1fg and CALSAMIGLIA *et al.* (1999) with a 80fg sensitivity.

The specificity test showed that only the ATCC *M. hyopneumoniae* strain present a predicted band of approximately 352bp. No other amplification fragment was observed using other microorganisms (Fig. 2).

Although *M. flocculare* and *M. hyopneumoniae* have shown high 16S rRNA sequence homology, primer sets used in the present study were highly specific for *M. hyopneumoniae* detection. Both 16S rRNA sequences were analyzed using BLAST program and resulted in 95% homology. The *M. hyopneumoniae* DNA region amplified by 352 bp N-PCR has four different base pairs when compared to *M. flocculare*. Thus, PCR primers for *M. hyopneumoniae* are complementary to a sequence that can be differentiated from *M. flocculare*, and are highly specific for *M. hyopneumoniae* detection (7).

The N-PCR amplified product was sequenced using *M. hyopneumoniae* specific primer and showed a 99% similarity

Tabela 3. Results obtained by N-PCR of samples collected from 40 pigs respiratory problems; considering sampling sites, sample transport media, and DNA extraction method.

Extraction	Transport media	Sampling site	Results		χ^2 *Test
			Positives N (%)	Negatives N(%)	
Boom	Saline	Tonsil	38 (95.0)	02 (05.0)	a
Boom	Saline	Nasal	32 (80.0)	08 (20.0)	ab
Fan	FRIIS	Tonsil	29 (72.5)	11 (27.5)	b
Fan	FRIIS	Nasal	06 (15.0)	34 (85.0)	c

*Statistical difference on standardization tests using the CONTRATE test χ^2 ($p < 5\%$) “Catmod SAS. Equal letters on the χ^2 *Test collumn indicate no significant difference.

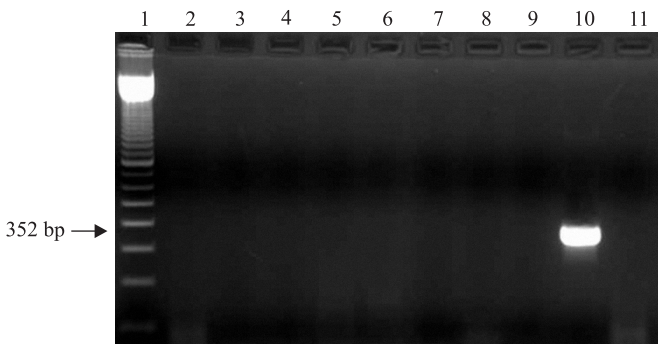


Figure 2. Specificity test results of N-PCR technique used for DNA detection of *M. hyopneumoniae* 16SrRNA gene. 1- 100 bp ladder (Amersham pharmacia biotech); 2- *Mycoplasma hyosynoviae*; 3- *Mycoplasma hyorhinis*; 4- *Mycoplasma flocculare*; 5- *Haemophilus parasuis* S1; 6- *Haemophilus parasuis* S2; 7- *Pasteurella multocida*; 8- *Actinobacillus pleuropneumoniae*; 9- *Bordetella bronchiseptica*; 10- *Mycoplasma hyopneumoniae*; 11- N-PCR negative control.

with the *M. hyopneumoniae* 16S rRNA sequence deposited in GenBank (Y00149).

The present study showed good N-PCR optimization results for partial detection of *M. hyopneumoniae* 16S rRNA gene from field samples. Biological samples obtained from tonsil swabs, stored in saline, and submitted to Boom DNA extraction method have shown superior results and are recommended as the best option for SEP diagnostic by NPCR. Moreover, they can be considered a promising method for monitoring *M. hyopneumoniae* infections in swine herds.

Specific media are indicated for the cultivation of mycoplasmas. In order to increase the sensibility of the culture technique some procedures may be followed: addition of antibiotics to the culture media, ten-fold serial dilutions (until 10^{-5}), and over two-week incubation period. However, these recommendations may increase the cost and the time necessary for a disease diagnostic. In this study, saline as transport media for clinical samples swabs resulted in a higher sensibility for mycoplasma detection when compared to specific mycoplasma media; thus, saline solution may be recommended as a transport media for the samples described herein.

RESUMO

Detecção do *Mycoplasma hyopneumoniae* pela reação em cadeia da polimerase em suínos apresentando problemas respiratórios

A Nested-PCR (N-PCR) tem como objetivo melhorar a sensibilidade do diagnóstico direto da Pneumonia Enzoótica

Suína, pois o isolamento do *Mycoplasma hyopneumoniae* é trabalhoso tornando-se inviável na rotina. Neste trabalho, foi realizado um projeto piloto para a otimização da técnica de N-PCR, utilizando três variáveis: tipo de amostra biológica, meio de transporte da amostra e método de extração do DNA, utilizando oito animais. Os resultados obtidos foram empregados no segundo experimento para a validação do teste utilizando 40 animais. Os resultados obtidos, pela otimização da N-PCR, neste trabalho, permite sugerir esta prova como método de diagnóstico de rotina no monitoramento das infecções por *Mycoplasma hyopneumoniae* em granjas de suínos.

Palavras-chaves: Pneumonia Enzoótica Suína, *Mycoplasma hyopneumoniae*, diagnóstico, Nested-PCR.

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