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# Polymerase Chain Reaction (PCR) for Detection of *Mycoplasma hyopneumoniae*, Responsable of Enzootic Pneumonia in Pigs

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**Abstract:** The ELISA test is generally used for the diagnosis of enzootic pneumonia caused by *M. hyopneumoniae*. This test only detects antibodies which can be found until 8 weeks after infection. The Polymerase Chain Reaction technique (PCR) has been developed in order to detect mycoplasma *in vivo*. It is a rapid test for diagnosis from nasal swab samples. In order to implement PCR in Mexico for the rapid detection of *M. hyopneumoniae* in pigs, a pure culture and two commercial vaccines were used. DNA extraction was performed with the reagent CTAB. One pair of primers that amplify a fragment of 352 bp of the *16Sr RNA* gene was used for DNA amplification. The sensitivity test showed that it can detect up to 3 pg of DNA. The specificity of the technique was also demonstrated when it did not amplified DNA from *M. bovis* and *P. multocida*. The technique was tested with nasal swab samples collected from 30 pigs of 12 weeks of age from three commercial farms with antecedents of respiratory disease. In one of the farms, 40% (4/10) samples were positive in the second, 20% (2/10) and the none in the third one (0/10). It is concluded that PCR was able to detect the *M. hyopneumoniae* from nasal swab samples from pigs with respiratory problems and represents a valuable tool for early diagnosis which will allow better control of the disease.

**Key words:** Enzootic pneumonia, *Mycoplasma hyopneumoniae*, diagnosis, PCR, pigs, Mexico

## INTRODUCTION

Enzootic pneumonia is caused by *Mycoplasma hyopneumoniae* and it is considered as the primary agent in chronic respiratory disease in pigs (Thacker, 2006). This disease is widely distributed in farms causing great economic losses to the swine industry due to an increase in medication and delayed growth of pigs (Ross, 1992).

*M. hyopneumoniae* is transmitted by direct nose-nose contact, colonizing and destroying the tracheobronchial ciliated epithelium thus, initiating infection and predisposing to infections by secondary respiratory pathogens. Gross lesions observed during infection consist of areas of pulmonary consolidation that are resolved 12-14 weeks after infection causing nonproductive cough, dyspnea and reduced pulmonary capacity, leading to a decrease in growth rate and feed efficiency in pigs chronically affected.

Sows are generally immune to *M. hyopneumoniae* and protect their litters for a period of 4-6 weeks of age. At the end of maternal immunity, *M. hyopneumoniae* begins to infect susceptible pigs. Infection is enhanced when piglets are subjected to weaning, sudden temperature changes, poor ventilation and overcrowding.

In the field, clinical signs with gross lesions observed in affected pigs can help make diagnosis (Thacker, 2004). However, diagnosis is complicated depending on the stage in which animals are infected. If the infection occurs at an early age the pulmonary lesions are resolved, showing only low weight so, the prevalence of the disease may be underestimated. Otherwise if the infection occurs in late stages of production, large number of lungs with lesions are seen but generally without weight loss of animals (Noyes *et al*., 1988; Scheidt *et al*., 1990). In the end, laboratory tests are necessary to reach a definitive diagnosis of *M. hyopneumoniae*.

Isolation of *M. hyopneumoniae* is a standard laboratory test but it is not routinely used because it is not easy to achieve (Thacker, 2006), besides being time-consuming as it requires 4-8 weeks for culture results and identification of the mycoplasma isolated (Maes *et al*., 1996). ELISA is the serological test that is

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commonly used to check the disease status of herds. This test is quick and easy to implement and is used to determine length of maternal immunity by serological profile, the possible age of infection and interactions with other agents making possible to establish control programs. However, antibodies can only be detect between 6-9 weeks after infection (Andreasen *et al*., 2004) causing the spread of *M. hyopneumoniae* amongst the herd.

In recent years different countries have established the Polymerase Chain Reaction technique (PCR), single or nested, for field diagnosis of *M. hyopneumoniae*. This technique has proven very useful for detecting microorganisms difficult to isolate in pigs such as *M. hyopneumoniae*. As the aim of this study was to establish the PCR technique for early diagnosis of enzootic pneumonia in swine herds in Mexico.

## MATERIALS AND METHODS

**Positive controls:** Two commercial vaccines against enzootic pneumonia and one culture of *M. hyopneumoniae* isolated from a clinical case were used as positive controls to carry out the establishment of the technique.

**DNA extraction:** It was performed by laboratory protocol using Cetyltrimethylammonium Bromide (CTAB). Prior to DNA extraction, 500 μL of culture of *M. hyopneumoniae* and 5 mL of each vaccine were centrifuged for 5 min at 13000 g, supernatants were discarded and the pellets were submitted to extraction. Each sample was added 400 μL of a TE solution (100 mM Tris-HCl pH 8.0 and 10 mM EDTA pH 8.0) plus 50 μL of lysozyme (10 mg $mL^{-1}$), vortexed and incubated 1 h at 37°C. Then, 75 μL of 10% SDS and 6 μL of proteinase K (10 mg $mL^{-1}$) were added, stirred and incubated 10 min at 65°C. After this, 100 μL of 5M NaCl solution and 100 μL of CTAB/NaCl solution (10/4.1%) were incorporated, stirred and incubated further 10 min at 65°C. About 750 μL of chloroform/isoamyl alcohol (24:1) solution were added, vortexed for 10 sec and centrifuged for 5 min at 12000 g. The supernatant was transferred in 200 μL volumes to a clean sterile tube being careful not to take the interface. Isopropyl alcohol was added to the volume transferred to 60% of the volume transferred. The sample was allowed to stand still for 30 min at -20°C and centrifuged for 15 min at 12000 g. The supernatant was discarded and the resulting DNA pellet was added 500 μL of 70% ethyl alcohol. The tube was gently inverted several times and centrifuged for 5 min at 12000 g. The supernatant was discarded carefully with a pipette and the pellet allowed to dry at room temperature for 45 min. The DNA was resuspended in 50 μL of water for injection. The tubes were placed in a water bath between 55-60°C for 5 min and frozen at -20°C until use.

**DNA amplification:** For this purpose a pair of oligonucleotides that amplify a fragment of 352 (bp) of 16S ribosomal gene were used, whose sequence is: 5'-ACTA GATAGGAAATGCTCTAGT-3'; 5'-GTGGACTACCAG GGTATCT-3 (Calsamiglia *et al*., 1999). The reaction mixture consisted of 1.5 mM $MgCl_2$, 200 μM of each deoxynucleotide, 1.25 U of Taq polymerase, 1X of reaction buffer, 20 pmol of each oligonucleotide and 1 μL of target DNA in a final volume of 25 μL. The mixture was subjected to a cycle of initial denaturation at 94°C for 4 min, 35 cycles at 94°C for 30 sec, 54°C for 30 sec, 68°C for 90 sec and a final extension cycle at 68°C for 3 min.

**Visualization of amplification products:** About 8 μL of each amplification product were analyzed by 1.5% gel electrophoresis in buffer Tris-Acetate-EDTA (TAE) dyed with ethidium bromide.

Tenfold dilutions were made of the culture DNA and tested by PCR in order to determine the sensitivity of the test. Moreover, to corroborate the specificity of the test, it was carried out with DNA from field isolates of *M. bovis* and *P. multocida*.

**Field samples:** An opportunity sampling was conducted in three commercial farms with antecedents of respiratory problems in fattening animals with the objective to test the technique with field samples. Ten nasal swabs were obtained from 12 weeks old pigs of each farm. The swabs were washed in 400 μL TE solution, DNA extraction and PCR were performed as previously described.

## RESULTS

Amplification of the expected product of 352 bp from the culture DNA and the two commercial vaccines was achieved (Fig. 1). The sensitivity test determined that the

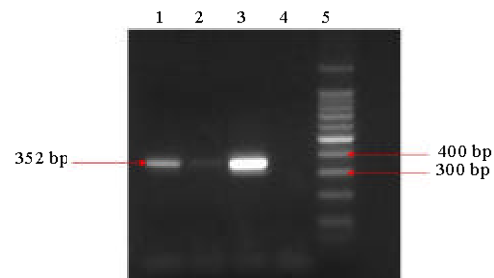


Fig. 1: PCR products obtained from two commerical vaccines and one culture of *M. hyopneumoniae*. Lane 1: Vaccine A; Lane 2: Vaccine B; Lane 3: Culture of *M. hyopneumoniae*; Lane 4: Negative control; Lane 5: Size marker

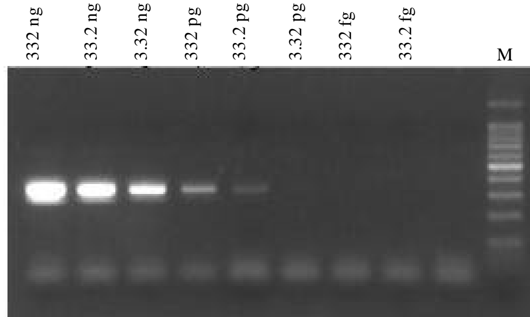


Fig. 2: PCR sensitivity assay for the detection of DNA isolated from *M. hyopneumoniae*

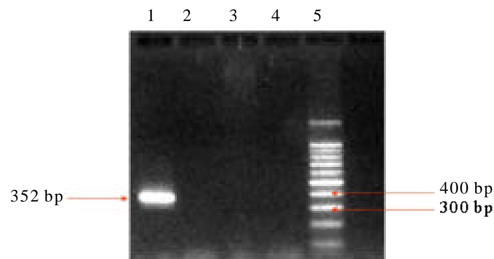


Fig. 3: Specificity PCR assay for detected of *M. hyopneumoniae*. Lane 1: *M. hyopneumoniae*; Lane 2: *M. bovis*; Lane 3: *P. multocida*; Lane 4: negative control; Lane 5: Size marker

technique was capable of detecting up to 3 pg of DNA (Fig. 2). Concerning the specificity of the test, there was no amplification when the PCR was performed from DNA of *M. bovis* or *P. multocida* (Fig. 3).

With regard to nasal swabs from pigs in the field in the first farm, 40% (4/10) samples were positive in the second, 20% (2/10) and none (0/10) was positive in the third farm.

## DISCUSSION

Because *M. hyopneumoniae* spreads rapidly in susceptible pigs is of great importance to have a diagnostic tool that is able to detect it early. This will allow the implementation of control measures to reduce economic losses due to increased medication and delayed growth. In this study, it was observed that under the conditions set for the amplification of the flanked sequence of the *16S rRNA* gene, it was possible to obtain the expected products from the DNA of *M. hyopneumoniae* culture and from the commercial vaccines against enzootic pneumonia. Through out the test sensitivity of the technique, it was determined that it was able to detect up to 3 pg of DNA. Other studies have found that setting a nested PCR increases the sensitivity up to 1 fg DNA, equivalent to between 5-50 organisms detected (Verdin *et al*., 2000; Kurth *et al*., 2002). However, in these trials, the likelihood of contamination and false positive results is elevated (Kwok and Higuchi, 1989). Regarding the specificity of the test, it was corroborated by evaluating a culture of *M. bovis* and *P. multocida* as well as the results of the negative controls.

Furthermore although, the number of field samples tested was small, 20% (6/30) of them were positive, a similar figure as reported in another study with samples from experimentally infected animals (Kurth *et al*., 2002). This can be explained by the fact that in infected animals, *M. hyopneumoniae* colonizes the ciliated epithelium of the trachea, bronchi and bronchioles (Blanchard *et al*., 1996) and it is not normally found colonizing the nasal cavity in healthy pigs (Goodwin, 1972).

## CONCLUSION

The results obtained in this study from nasal swab samples may suggest early infection in animals as has been reported that PCR can detect *M. hyopneumoniae* in the 1st week after infection, compared with the culture isolation technique which is able to detect it until the 2nd week. Thus, it is a valuable diagnostic tool due to its sensitivity and specificity for *M. hyopneumoniae* detection in nasal swabs in the early stages of the disease and/or apparently healthy carriers that are an important source of infection to susceptible pigs. The PCR technique will be useful for early diagnosis of enzootic pneumonia in pig farms which will help avoid the negative effects associated with the disease control by implementing appropriate measures to prevent transmission within and outside the farm.

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