Development of In Situ Hybridization for the Detection of Mycoplasma hyorhinis in Formalin-Fixed Paraffin-Embedded Tissues from Naturally Infected Pigs with Polyserositis

Bongtai KIM1)**, Kichan LEE1)**, Kiwon HAN1), Duyeol KIM1), Yooncheol HA1), Chung Hyun KIM1), Yeonsu OH1), Iikje KANG2), Jeheoon LEE2) and Chanhee CHAE1)*

1)Department of Veterinary Pathology, College of Veterinary Medicine, Seoul National University, 599 Gwanak-ro, Gwanak-Gu 151–742, Seoul and 2)Dong Bang Co., Ltd., Dogok-Dong 517–8, Gangam-gu, Seoul 135–272, Republic of Korea

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Abstract. The aim of this study was to develop in situ hybridization for the detection of Mycoplasma hyorhinis in formalin-fixed, paraffin-wax-embedded tissues from pigs with polyserositis. M. hyorhinis was isolated from the spleen (2 pigs) and pericardium (1 pig). M. hyorhinis DNA was detected 16 out of 20 pigs with polyserositis. In situ hybridization produced a distinct positive signal for the M. hyorhinis p37 gene in inflammatory cells in the polyserositis. In situ hybridization developed in the present study present diagnostic tools capable of detection of M. hyorhinis in formalin-fixed, paraffin-wax-embedded tissues from the naturally infected pigs.

Key words: in situ hybridization, Mycoplasma hyorhinis, swine.

In situ hybridization is a valuable research and diagnostic tool, especially when it is necessary to detect M. hyorhinis in formalin-fixed, paraffin-wax-embedded tissues although the labor and time required for in situ hybridization are prohibitive for many laboratories. The objective of this study was to develop for detection of M. hyorhinis DNA in naturally infected pigs.

Samples were obtained at necropsy from pigs submitted to the Department of Veterinary Pathology of Seoul National University between July 2008 and June 2009. Tissue samples (lung, liver, pericardium, spleen and lymph node), after 1 or 2 days’ fixation in neutral buffered 10% formalin, were dehydrated through graded alcohols and a xylene step and embedded in paraffin wax for histopathological examination of haematoxylin and eosin (HE)-stained sections (4 μm). Twenty pigs were selected from 143 cases on the basis of polyserositis and no bacterial isolation such as S. suis and H. parasuis.

Five colostrum-deprived pigs aged 35 days were used as a source of negative control tissues. Lung sections from pigs naturally infected with Pasteurella multocida capsular type A and pigs experimentally infected with Actinobacillus pleuropneumoniae and M. hyopneumoniae, were also used to provide further control material. These lung sections were shown to be free of M. hyorhinis by examining polymerase chain reaction (PCR) amplified DNA extracts from the corresponding lung samples.

All 20 pigs were humanely euthanized by electrocution for necropsy. Specimens of the lung, liver, pericardium and spleen were used for isolation of M. hyorhinis. The mycoplasma was isolated from tissue samples as previously described [7, 10]. Field isolates were identified as M. hyorhinis by PCR [5]. Field isolates were grown in Friis medium and harvested at pH 6.9. The mycoplasma suspension was centrifuged (20,000 × g) and washed three times in
0.25 M NaCl. Genomic DNA was purified using the Rapid-prep microgenomic DNA isolation kit (Pharmacia Biotech, Uppsala, Sweden) following the manufacturer’s instructions and performed PCR as previously described [5].

A 357 base pair (bp) fragment of the p37 gene of *M. hyorhinis* (GenBank accession number X14140) was used as a probe and carried out as previously described [5]. The forward and reverse primers were 5’-GTAGTCAAGCAGAGGATGT-3’ and 5’-GCTGGAGTTATTATACGGAGA-3’, respectively.

PCR products were purified with a 30-kD “cut-off” membrane filter. The nucleotide sequences of the purified PCR products were determined by BigDye chemistry with the ABI Prism Sequencer (Applied Biosystems, Foster City, CA, U.S.A.). Sequencing was performed on the purified PCR products before PCR products were labelled by random priming with digoxigenin-dUTP (Boehringer Mannheim, Indianapolis, IN, U.S.A.) according to the manufacturer’s instructions. The *M. hyopneumoniae* probe was prepared as previously described [11] for negative control purposes. *In situ* hybridization was performed as previously described [9].

*M. hyorhinis* was isolated from the spleen (2 pigs) and pericardium (1 pig). *M. hyorhinis* DNA was detected 16 out of 20 pigs with polyserositis. Histopathologically, fibrinous inflammation was observed in the serous membrane of the heart (Fig. 1), peritoneum, lung, spleen, liver, and kidney. The morphology of the tissues was well-preserved, despite the relatively high temperature required for the incubation procedure. *In situ* hybridization produced a distinct positive signal for the *M. hyorhinis* p37 gene in the polyserositis. The intensity and extent of labelling for *M. hyorhinis* were detected in fibrous inflammatory area of polyserositis (Fig. 2). Strong hybridization signals for *M. hyorhinis* were detected in the inflammatory cells in polyserositis. In those areas, identification of the cell types containing *M. hyorhinis* p37 gene was occasionally difficult, but examination of adjacent sections stained with HE confirmed that positive cells resembled neutrophil-like cells, which had bilobed nuclei.

Pretreatment with DNase I eliminated hybridization signals from the 16 samples naturally infected with *M. hyorhinis*. Sections of the lung from the negative control pigs, the pigs naturally infected with *P. multocida* capsular type A, and the pigs experimentally infected with *A. pleuropneumoniae* and *M. hyopneumoniae* showed no hybridization signals for *M. hyorhinis* and *H. parasuis*. Moreover, the DNA probes for *M. hyopneumoniae* were consistently negative in all lung, heart, spleen, liver, and lymph node tissues from...
naturally infected pig.

The study demonstrated that *M. hyorhinis* can be detected and differentiated in formalin-fixed, paraffin-wax-embedded tissue specimens of infected pigs by means of a non-radioactive labelled DNA probe. Hybridization signals for *M. hyorhinis* were identified in neutrophils in the polyserositis. The diagnosis of *M. hyorhinis* is usually done by cultivation of the organisms. However, due to the fastidious nature of *M. hyorhinis*, its culture may take up to 1 month [7, 9]. Since cross-reactions which have been reported between *M. hyopneumoniae* and *M. hyorhinis* [1, 2, 6], serological and immunohistochemical methods are hampered to differentiate these mycoplasma species. Therefore, in situ hybridization is an alternative method. The DNA probes for *M. hyorhinis* were not hybridized consistently in *M. hyopneumoniae*-infected tissues and vice versa. Therefore, the probes of *M. hyorhinis* developed in this study could be specific for detection of *M. hyorhinis* in formalin-fixed, paraffin-wax-embedded tissues.

In situ hybridization using a fluorescein-labelled probe had been reported for the differentiation between *M. hyopneumoniae* and *M. hyorhinis* in formalin-fixed tissues [3]. Although *M. hyorhinis* DNA can be detected in cells by fluorescent in situ hybridization, definitive localization of the mycoplasma DNA within various cells has not been possible, especially when the amount of DNA is small. In addition, use of a fluorescence microscope is necessary, and the stained slides are not suitable for permanent recording keeping. In contrast, in situ hybridization using a digoxigenin-labeled probe is able to determine which cells, in a mixed population, or tissues are expressing the DNA of interest. In situ hybridization developed in the present study represents a diagnostic tool capable of detection of *M. hyorhinis* in formalin-fixed, paraffin-wax-embedded tissues from naturally infected pigs.