# *Mycoplasma hyorhinis* Infection Levels in Lungs of Piglets with Porcine Reproductive and Respiratory Syndrome (PRRS)

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ABSTRACT. The infection levels of *Mycoplasma hyorhinis*, *M. hyopneumoniae* and *M. hyosynoviae* in the lung of piglets were examined in relation to porcine reproductive and respiratory syndrome (PRRS). These animals consisted of 43 PRRS piglets with PRRS, 2 piglets infected with PRRS virus but symptom-free, and 10 control piglets free of PRRS virus and its antibody. *M. hyorhinis* was isolated from 40 of the 43 PRRS piglets, from 1 of the 2 latent infected piglets and from 3 of the 10 control piglets. The number of *M. hyorhinis* isolated from the lungs of PRRS piglets was more than $10^5$ CFU/g, but those isolated from the latent infected piglets and the control piglets were less than $10^3$ CFU/g. In addition to this, *Haemophilus parasuis* and *Pasteurella* spp. were frequently isolated from the piglets with PRRS (51.2% and 25.6%, respectively). On the other hand, *M. hyopneumoniae* was isolated from only 4 of 55 piglets tested, and *M. hyosynoviae* was not isolated. *M. hyorhinis* was also detected directly in the lung emulsion samples from almost all the PRRS piglets using a polymerase chain reaction-based method.—KEY WORDS: mycoplasma, *Mycoplasma hyorhinis*, PCR, PRRS, swine.

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Porcine reproductive and respiratory syndrome (PRRS) was reported as a new pig disease in the eastern part of the Netherlands in the beginning of 1991 [11, 13]. Actually, this disease seemed to have existed in countries with a modern pig industry, e.g. the United States, Canada, and many European countries including the Netherlands prior to this [9]. In 1993, we succeeded in isolating PRRS virus for the first time in Japan from young swine that showed severe abdominal breathing [9]. Although it has been confirmed that a virus isolated from the field can cause pneumonia in hysterectomy-produced and colostrum-deprived (HPCD) piglets, the severity of illness in these piglets seemed to be much milder than that in the field cases [9]. It is believed that PRRS co-occurs with other microorganisms [9]. Several bacteria have been isolated, such as *Streptococcus* spp., *Pseudomonas* spp., and *Escherichia coli*, etc. [2]. Wensvoort *et al.* isolated mycoplasmas (the trivial name for microorganisms belonging to the class *Mollicutes*), such as *Acholeplasma laidlawii*, *Mycoplasma hyopneumoniae*, and *M. hyosynoviae* from nasal swab samples of sows [13]. However, those bacteria were isolated only sporadically.

On the other hand, we have found a considerable amount of *M. hyorhinis* to be common in the lungs of 1- to 2-month-old piglets with PRRS [9]. *M. hyorhinis* is one of the mycoplasmas that grows well in culture and can be easily isolated when an adequate mycoplasmal medium is used. However, the mycoplasmal media cannot be easily applied for clinical identification in the field because of the difficulty in preparing the media. Moreover, even if some mycoplasmas were isolated by the cultivation method, it is time-consuming to identify them with their antisera. In this study, we examined the infection levels of *M. hyorhinis* and other bacteria in the lungs of PRRS piglets. Moreover, we attempted to detect mycoplasmas (*M. hyorhinis*, *M. hyosynoviae*, and *M. hyopneumoniae*) in lung emulsion samples from PRRS piglets using a polymerase chain reaction (PCR)-based method.

## MATERIALS AND METHODS

*Samples of piglets*: Fifty-five piglets (21 to 84 days old) were obtained from 5 farms in the eastern part of Japan. Forty-three of the piglets showed severe abdominal breathing and lost weight. The 12 remaining, apparently healthy piglets were obtained from two of 5 farms.

*Diagnosis of PRRS virus infections*: Lung emulsion samples or lingual tonsils of piglets were tested for PRRS virus, and the sera were examined for antibodies against PRRS virus according to Shimizu *et al.* [9]. When neither the PRRS virus nor its antibody was detected in a symptom-free piglet, the piglet was designated as a PRRS-free control animal.

*Media and bacterial isolations*: For the isolation of bacteria such as *Haemophilus* spp., *Actinobacillus* spp., *Pasteurella* spp., and *Streptococcus* spp., Trypticase Soy agar medium (YHT medium) supplemented with 1% fresh yeast extract and 5% horse blood was employed. The lung sample was emulsified and diluted in a series of 10-fold dilution with antimicrobial agent-free BHL broth [16]. Subsequently, 10 $\mu l$ of each dilution was dropped onto YHT medium. The other samples (the liver, kidney, spleen, heart, and brain) were stamped directly onto YHT medium. Plates were incubated aerobically at 37°C with or without 5% $CO_2$ gas for 48 hr. Isolates were identified by biochemical and serological examinations when they could significantly grow on the plate ($10^4$ CFU/g or more per sample).

In order to isolate mycoplasmas from the samples, 2 kinds of swine mycoplasmal media were employed. For isolation

of *M. hyorhinis* and *M. hyosynoviae*, 0.5% (w/v) mucin-added PPLO broth medium (M broth) [7, 15] was used. Modified BHL broth medium for selective isolation of *M. hyopneumoniae* was prepared by adding a rabbit hyper antisera against *M. hyorhinis* strain BTS7 at a final concentration of 2.0% to suppress the growth of *M. hyorhinis* in the medium. M agar and BHL agar media were prepared by adding 1.0% of noble agar (Difco) to each broth medium. Serial 10-fold dilution of the lung emulsions were prepared to $10^{-6}$ in the BHL broth and each 10 $\mu l$ portion was dropped onto M agar. The other samples described above were stamped directly onto M agar. Duplicate plates were incubated aerobically with 5% $CO_2$ for isolation of *M. hyorhinis*, and anaerobically for isolation of *M. hyosynoviae*, at 37°C for 5 days. Moreover, the M broth was inoculated with lung emulsion as enrichment culture of those swine mycoplasmas and incubated at 37°C for 2 or 3 days, then a loop of the culture was spread on M agar and the plates were incubated aerobically or anaerobically at 37°C for 5 days. The primary mycoplasmal isolates were purified three times by using a micropipet to suck an agar plug bearing one colony. The procedure of isolation of *M. hyopneumoniae* was conducted, using BHL medium described previously [16].

*Identification of mycoplasmas*: Mycoplasmal isolates were tested for biochemical properties such as cholesterol requirement (an indirect method based on digitonin sensitivity), glucose digestion, arginine hydrolysis, and tetrazolium reduction [1]. Then the mycoplasmas were serologically identified by metabolism inhibition test [10].

*Test procedures of PCR*: The sequence of the primers used in the polymerase chain reaction (PCR) for the detection of swine mycoplasmas was constructed on the basis of the variable regions in 16S ribosomal ribonucleic acid (rRNA) dominant gene of each swine mycoplasma species [3, 4, 12]. The sequence of PCR primers and the test procedures were illustrated in Fig 1. Prior to PCR examination, the lung samples were treated as follows: 0.5 g of each lung sample was emulsified with 2.0 m*l* of phosphate-buffer saline (PBS), and 1.0 m*l* of the lung emulsion was transferred to a new eppendorftube. After the lung emulsion sample had been spun at 2,000 rpm for 5 min, the supernatant was centrifuged at 15,000 rpm, for 5 min. The pellet was suspended in lysis solution which contained protainase K (Sigma) and CHAPS (Wako Pure Chem.), and the mixture was incubated at 60°C for 30 min and subsequently at 94°C for 5 min (Fig. 1). Five $\mu l$ of the resulting mixture was used as template DNA for the PCR. The PCR amplification was performed in a 50 $\mu l$ reaction mixture containing 50 pM each of the oligonucleotide primers, 1 mM each of the four dNTP, and 1.25 units of Taq-DNA polymerase. The reaction mixture was overlaid with mineral oil and subjected to 30 cycles of amplification in a programmable heating block (Hoei Science, Tokyo). The program used was as follows: 94°C, 30 sec for denaturation, 60°C, 30 sec for annealing, and 72°C, 60 sec for extension. After amplification, 5 $\mu l$ of the PCR products was analyzed on a 1.2% agarose gel (Sigma, Type II) by electrophoresis in Tris-acetate-EDTA buffer. Electrophoresis was carried out at 80 V for 1–1.5 hr, and then the gel was stained with a solution containing 1.0 $\mu g$ of ethidium bromide per m*l* for 15 min. DNA fragments were visualized by UV illumination at 312 nm.

*PCR detection of mycoplasmas in simulated samples*:

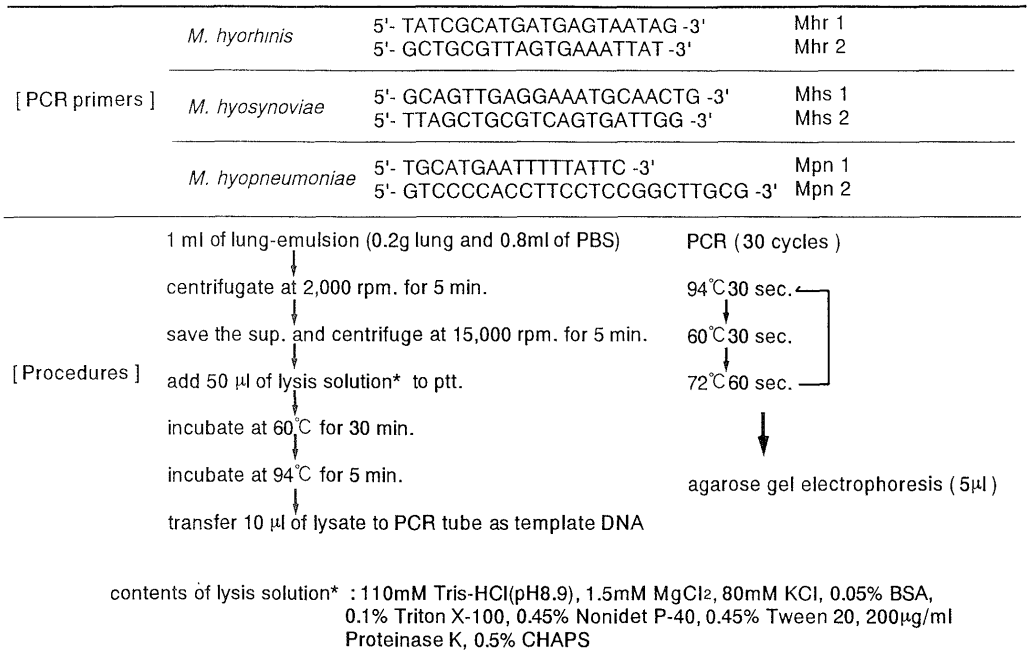


Fig. 1. DNA primers employed for detection of swine mycoplasmas and their procedures.

Mycoplasma-free piglet lung emulsions were used. These emulsions were diluted five times in a physiological salt solution and infected with known amounts of broth-grown mycoplasmas, defined by determining the numbers of CFU. The PCR procedure was the same as described above.

## RESULTS

*Piglets*: PRRS virus was isolated from lung or lingual tonsil samples of 34 out of 43 piglets that showed severe abdominal breathing, but the virus could not be isolated from the remaining 9 piglets that had an antibody against PRRS virus. On the other hand, 2 out of 12 piglets without any symptoms were also infected with PRRS virus. The remaining 10 piglets, which had neither PRRS virus nor its antibody, were designated as controls. Pathologically, proliferative and interstitial pneumonia was recognized in all lung lobes of the 43 PRRS piglets.

*Isolation of mycoplasmas*: More than $10^5$ CFU/g of *M. hyorhinis* was isolated from the lungs of 40 out of 43 (93.0%) piglets that showed severe abdominal breathing (Table 1). On the other hand, less than $10^3$ CFU/g of *M. hyorhinis* was isolated from 1 of the 2 latent infected piglets and from 3 of the 10 control piglets. *M. hyorhinis* was also isolated from the other organs in half of the piglets with PRRS by culturing them onto M agar. The isolation rate of *M. hyorhinis* was 20 out of 43 (46.5%) from the kidney, 10 out of 43 (23.3%) from the heart, and 10 out of 43 (23.3%) from either the brain or the liver. The number of colonies of *M. hyorhinis* detected on M agar ranged from approximately 1 to $10^4$ CFU. *M. hyopneumoniae* was isolated from the lung samples from 4 of 55 piglets tested. This organism was isolated not only from three PRRS piglets but also from one of PRRS-free piglets. An arginine-utilizing mycoplasma was isolated from only one lung sample of the piglets with PRRS by enrichment culture under anaerobical incubation. It was identified as *M. arginini* by a metabolism inhibition test using the rabbit antiserum. However, *M. hyosynoviae* was not isolated from any piglets.

Table 1. Comparative infection levels of *M. hyorhinis* between PRRS and PRRS-free piglets

| Infection level of *M. hyorhinis* in lung sample (CFU/g) | PRRS | |
|---|---|---|
| | +[a] | − |
| >1[b] × $10^8$ | 8[c] (8)[d] | 0 |
| 1 × $10^7$ | 22 (22) | 0 |
| 1 × $10^6$ | 5 (5) | 0 |
| 1 × $10^5$ | 5 (5) | 0 |
| 1 × $10^4$ | 0 | 0 |
| 1 × $10^3$ | 1 (0) | 3 (0) |
| <1 × $10^2$ | 4 (3) | 7 (0) |

a) Positive in PRRS virus or its antibody.
b) Coefficient rounded to real number.
c) Number of piglet.
d) Number of piglet showing severe abdominal breathing.

*Isolation of non-mycoplasmal bacteria*: *H. parasuis* was isolated from the lung samples of 22 out of 43 (51.2%) piglets with PRRS (Table 2). Also, *Pasteurella* spp. were rather frequently isolated (11/43, 25.6%). In some cases, mixed contamination with *H. parasuis* and *Pasteurella* spp. were observed. *Pseudomonas* spp. (2/43, 4.7%) and *Escherichia coli* (1/43, 2.3%) were purely isolated on YHT medium. All lung samples from which these bacteria were isolated to a significant degree using YHT medium (more than $10^4$ CFU/g in a lung sample) were contaminated with a considerable number of *M. hyorhinis* (Table 2). These bacterial counts in the lungs of the piglets with PRRS were higher than those in the lungs of the piglets without PRRS symptoms. No bacteria could be significantly isolated from either abdominal or thoracic organs.

*Mycoplasmal detection by PCR*: The specificity and sensitivity of the PCR experiments using simulated lung emulsion samples are shown in Fig. 2. *M. hyopneumoniae* (type strain J) could not be detected by its primers until its count was more than $10^5$ CFU per reaction tube when the

Table 2. Co-levels of *M. hyorhinis* and other bacteria found in lungs of PRRS and PRRS-free piglets

| Infection level of *M. hyorhinis* in lung sample (CFU/g) | No. of piglet | Bacterial species isolated from lung | | | | |
|---|---|---|---|---|---|---|
| | | *H. parasuis* | *Pasteurella* spp. *P. multocida* | | *Pseudomonas* spp. | *Escherichia coli* |
| >1[a] × $10^8$ | 8 | 3[b] | 1 | 2 | 0 | 0 |
| 1 × $10^7$ | 22 | 12 | 2 | 3 | 0 | 0 |
| 1 × $10^6$ | 5 | 4 | 3 | 0 | 1 | 0 |
| 1 × $10^5$ | 5 | 3 | 0 | 0 | 1 | 1 |
| 1 × $10^4$ | 0 | 0 | 0 | 0 | 0 | 0 |
| 1 × $10^3$ | 4 (3)[c] | 0 | 0 | 0 | 0 | 0 |
| <1 × $10^2$ | 11 (7) | 0 | 0 | 0 | 0 | 0 |
| Total | 55 | 22 | 6 | 5 | 2 | 1 |

a) Coefficient rounded to real number.
b) Number of piglet significantly infected with bacteria except *M. hyorhinis* (more than $10^4$ CFU/g in lung sample).
c) Number in parentheses indicating the number of piglet used as PRRS-free control.

simulated lung emulsion was tested. However, *M. hyorhinis* (type strain BTS7) and *M. hyosynoviae* (type strain S16) were detected by their primers when the reaction tubes contained more than $10^3$ CFU of these organisms. Each of these mycoplasmas was detected only by its own primers.

The results of detecting *M. hyorhinis* from the lung emulsions of the piglets with PRRS by PCR are shown in Table 3. This PCR method could detect *M. hyorhinis* from the lung emulsion samples when the number of *M. hyorhinis* was more than $10^3$ CFU per reaction tube ($10^4$ CFU/g or more per sample). When infection levels ranged from $10^2$ to $10^3$ CFU/g in the lung samples, the PCR products could be detected in 2 out of 10 samples. On the other hand, no *M. hyopneumoniae* could be detected by PCR even from 4 lung samples from which this organism had been isolated by the cultivation method. *M. hyosynoviae* was not detected from any piglets by PCR.

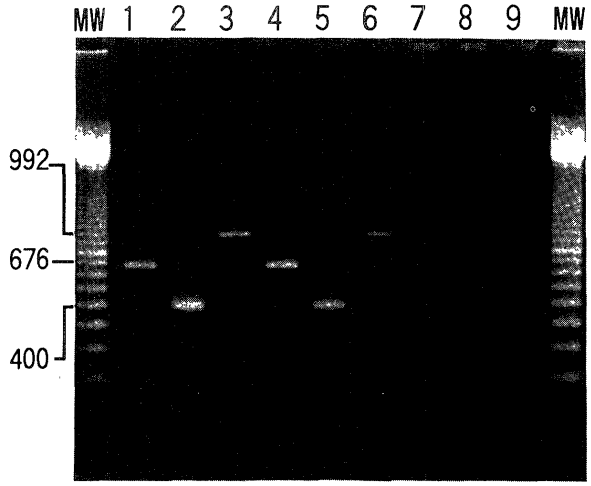


Fig. 2. Agarose gel electrophoregram of PCR-amplified products stained with ethidium bromide. Lanes 1, 2 and 3 were loaded with 5 $\mu l$ samples of amplified products from purified total DNAs of *M. hyorhinis* BTS7 (2.5 *ng*), *M. hyosynoviae* S16 (2.5 *ng*), and *M. hyopneumoniae* J (2.5 *ng*), respectively. Lanes 4, 5, and 6 were loaded with 5 $\mu l$ of amplified-products from simulated lung emulsion samples. Each simulated sample contained approx. $10^6$ CFU/m*l* of one of the three mycoplasma species. Lanes 7, 8, and 9 were loaded with 5 $\mu l$ from the reaction, using 2.5 *ng* DNAs from, *P. multocida* kobe 6, *H. parasuis* ATCC19417, and *E. coli* V517, respectively, as a template. Primers used were as follows; *M. hyorhinis* (lanes 1, 4, 7), *M. hyosynoviae* (lanes 2, 5, 8), and *M. hyopneumoniae* (lanes 3, 6, 9). Molecular weight standards (MW) were run simultaneously and the sizes of the fragments are given in bases on the left.

## DISCUSSION

The natural habitat of *M. hyorhinis* is the respiratory tract, especially the nasal cavity, of piglets [14]. Until about 1986, *M. hyorhinis* was considered non-pathogenic [14]. Gois *et al.* reported that several strains of *M. hyorhinis* induced pneumonia in gnotobiotic piglets exposed via the respiratory tract, and other researchers reported pathogenicities of these organisms, such as arthritis, serositis and a primary role in some outbreaks of piglet pneumonia [14]. Recently, Kazama *et al.* [5] suggested that *M. hyorhinis* is a primary causal agent of otitis media in pigs. The pathogenicity of *M. hyorhinis* may vary with the strain. In our experiments [9], several strains of *M. hyorhinis* isolated from the lungs of piglets with PRRS did not induce pneumonia in gnotobiotic piglets by intranasal inoculation. However, we considered that the assistant role of *M. hyorhinis* in inducing a serious pneumonia in combination with PRRS virus to be an important one, because the serious pneumonic consequence seems not to be induced by PRRS virus alone. Furthermore, the lung samples of 40 out of 43 piglets with PRRS were associated with a considerable number of *M. hyorhinis* leveling at least $10^5$ CFU/g. In contrast, those of the PRRS-free piglets were at levels less than $10^3$ CFU/g.

On the other hand, *M. hyopneumoniae* was isolated from 4 lung samples only. Moreover, the results of the PCR examination, however suggested that the infection was at a level less than $10^6$ CFU/g per lung sample (Fig. 2). These date suggest that *M. hyopneumoniae* play unimportant role in the pathogenesis of PRRS.

It has been reported that pigs over three months old were easily infected with *M. hyosynoviae* from contact with infected pigs, but that pigs less than this age were not so

Table 3. Detection of *M. hyorhinis* from lung samples of PRRS piglets by PCR or cultivation

| Infection level of *M. hyorhinis* in lung sample (CFU/g) | No. of lung sample[a)] | Detected by | |
|---|---|---|---|
| | | PCR | Direct cultivation[b)] |
| $>1 \times 10^8$ | 5 | 5 | 5 |
| $>1 \times 10^7 - 1 \times 10^8$ | 5 | 5 | 5 |
| $>1 \times 10^6 - 1 \times 10^7$ | 5 | 5 | 5 |
| $>1 \times 10^5 - 1 \times 10^6$ | 5 | 5 | 5 |
| $>1 \times 10^2 - 1 \times 10^3$ | 10 | 2 | 10 |
| $\leqq 1 \times 10^2$ | 10 | 0 | 2[c)] |

a) Including lung samples from PRRS-free piglets.
b) Lung sample was stamped onto M-agar medium.
c) *M. hyorhinis* isolated by enrichment culture.

infected [8]. In the present study, *M. hyosynoviae* could not be detected neither by cultivation nor by PCR. As yet it is unknown why *M. hyosynoviae* cannot infect very young pigs.

*H. parasuis* and *Pasteurella* spp. were rather frequently isolated from the lungs of PRRS piglets. These organisms might co-infect the PRRS piglets with *M. hyorhinis* for they were always found to co-occur with a considerable number of *M. hyorhinis*. On the other hand, *M. hyorhinis* was found to occur alone in several lung samples of PRRS piglets.

Our results suggest that PCR has significant potential as a rapid, sensitive tool for detecting and identifying *M. hyorhinis* in the lungs of PRRS piglets. We used proteinase K and CHAPS in the PCR because both reagents were considered to be very useful for extracting mycoplasmal DNAs and for digesting harmful proteins. The least infection level of *M. hyorhinis* in the lungs of PRRS piglets was at $10^5$ CFU/g and this organism was detected in the lungs of those piglets by PCR (Tables 1 and 3). In contrast, *M. hyorhinis* was not detected in the lungs of PRRS-free control animals by PCR. When PRRS virus or its antibody is detected from the lungs or sera of piglets that show severe abdominal breathing, the lung samples should be examined for the exsistence of *M. hyorhinis* by PCR because such animals might have been infected with *M. hyorhinis*. Moreover, our results indicate that PRRS viral isolates may be easily contaminated with *M. hyorhinis* through the isolation of the virus. Therefore, it is necessary to add effective antibiotics so that inhibit the growth of *M. hyorhinis* during the isolation of PRRS virus.

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