

Detection of *Mycoplasma hyopneumoniae* in Lung and Nasal Swab Samples from Pigs by Nested PCR and Culture Methods

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ABSTRACT. We examined nasal swab and lung homogenate samples collected from pigs experimentally and naturally infected with *Mycoplasma hyopneumoniae* for the detection of *M. hyopneumoniae* by the nested PCR (nPCR) and culture methods. In the 23 experimentally infected pigs, *M. hyopneumoniae* was commonly detected in nasal swabs by the nPCR and culture methods at 4 weeks after inoculation, and there was a significant correlation ($P < 0.01$) between the titers of viable organisms in nasal swabs and in lung homogenates in the experimentally inoculated pigs. In the naturally infected pigs, on the other hand, discrepancies in detection were found between nasal swab and lung homogenate samples in 17 of 36 cases, although the presence of gross lung lesions correlated relatively well with the detection of organisms from the samples. Our results indicated that the diagnosis of mycoplasmal pneumonia by nPCR in individual pigs with nasal swabs is reliable under these experimental conditions. At present, nPCR with nasal swabs should only be used for monitoring the disease status at the herd level under field conditions.

KEY WORDS: diagnosis, *Mycoplasma hyopneumoniae*, nasal swab, nested PCR, swine.

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Mycoplasma pneumoniae of swine (MPS) caused by *Mycoplasma hyopneumoniae* is one of the most common and economically important diseases found at pig farms worldwide, having low mortality but high morbidity. The clinical signs and lung lesions of MPS are not always characteristic under field conditions, implying a combination of many other factors such as microorganisms, stress, and environmental conditions. Isolation of the microorganism by the culture method with clinical samples from lung lesions is considered the standard technique for the diagnosis of MPS, but because of the slow growth of *M. hyopneumoniae* and the risk of overgrowth of *M. hyorhinis*, which is frequently isolated from the porcine lung, detection of *M. hyopneumoniae* using this method is not actually suitable for this purpose [15]. The recent introduction of polymerase chain reaction (PCR) assays has provided an alternative to the culture method, facilitating rapid, specific, and sensitive detection of *M. hyopneumoniae* [1, 3, 5, 10, 12]. Recent work has documented the usefulness of the nested PCR (nPCR) method, which has improved sensitivity for the detection of *M. hyopneumoniae* from bronchoalveolar lavage (BAL) fluids [11, 19], nasal swabs [6–8, 16] obtained from live pigs, and air samples [18] from pig houses to monitor the disease in the herd. BAL fluids obtained from live pigs as clinical samples for nPCR were very useful [11], except for the difficulty of technical procedures and the burden on the pig. Anesthesia and BAL are laborious and impractical under field conditions since BAL fluids are usually recovered from animals under the general anesthesia

[14]. On the other hand, the use of nasal swab samples for nPCR is suitable for detection of *M. hyopneumoniae* in routine clinical practice because of the ease of sample collection [6]. However, the relative advantages of the use of nasal swab samples from naturally infected pigs in the field for nPCR has not been fully established.

In this study, we have investigated the suitability of nasal swab samples as an alternative to lung samples from pigs experimentally and naturally infected with *M. hyopneumoniae*, and compared this procedure with the culture method using nPCR for the detection of *M. hyopneumoniae*.

MATERIALS AND METHODS

Experimental infection: Twenty-three 4-week-old cesarean section-derived, colostrum-deprived (CDCD) pigs, produced in our laboratory and maintained in a pathogen-free environment with controlled air temperature, were used. All pigs were intranasally inoculated with 10^6 color-changing units (CCU) of *M. hyopneumoniae* E-1 [13], as described previously [14]. Nasal swab samples were collected from 6 of the 23 inoculated pigs by inserting cotton swabs deep into the nasal cavities prior to inoculation, and 1, 2, and 3 weeks post-inoculation (PI). Each swab was placed in a tube containing 1 ml of sterile PBS and stored at -80°C until use. At four weeks PI, nasal swab samples were collected from all pigs. All pigs were euthanized by exsanguination after deep anesthesia with sodium pentobarbital (25 mg/kg; ABOTT, U.S.A.), and then necropsied. The percentage of the lung area occupied by lesions was calculated using an image processor (Qube 600, Nexus Co. Ltd., Tokyo) as described previously [2]. After macroscopic assessment, lung tissue from lung lesions typical for MPS were aseptically collected and

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Table 1. Longitudinal study of *M. hyopneumoniae* in nasal swabs collected from 6 experimentally inoculated pigs by the culture method and nPCR

	No. of nasal swab samples positive for <i>M. hyopneumoniae</i> (%)				
	Prior to inoculation	1 week PI	2 weeks PI	3 weeks PI	4 weeks PI
Culture	0 (0)	0 (0)	1 (16.7)	3 (50.0)	6 (100)
nPCR	0 (0)	5 (83.3)	2 (33.3)	4 (66.7)	6 (100)

PI: Post-inoculation.

were then homogenized in a BHL medium [20] to make a 10% suspension (w/v). Nasal swabs and lung homogenates obtained from experimentally inoculated pigs were tested for *M. hyopneumoniae* in parallel by the culture method and nPCR.

Clinical specimens: To evaluate the detection of *M. hyopneumoniae* by nPCR from nasal swabs under field conditions, clinical specimens were collected from 36 fattening pigs on farms with a history of chronic pneumonia. Nasal swabs were collected prior to slaughter at the abattoirs. Then, the lungs were examined macroscopically, and lung samples were collected from the same pigs. In pigs having no lesions, samples were taken from the apical lobes of the right lung. Lung homogenates were obtained in the same manner as for experimental infections. *M. hyopneumoniae* was detected in the nasal swabs by nPCR, and was isolated from lung homogenates by the culture method.

Mycoplasma culture: Mycoplasma culture was performed in a BHL medium as described previously [17]. Samples were diluted in a series of 10 fold-dilutions with medium and incubated at 37°C for up to 4 weeks. Isolates were cloned three times before being identified. Cultures were identified as *M. hyopneumoniae* by a PCR test as described previously [12].

Nested PCR: DNA from 100 µl of each sample was extracted using a commercial kit (WB kit, Wako) according to the manufacturer's instructions. Nested PCR was performed as described previously [6], with several modifications. Briefly, 2 µl of the DNA preparation was used as the PCR template in the first reaction, and 0.5 µl of PCR product was used for the second PCR. The amplification was performed in a 50 µl reaction mixture containing 0.2 nmol of each primer, 1 mM of dNTPs, 10 × PCR buffer, 2 mM of MgCl₂, and 2.5 U of TaqDNA polymerase (EXTaq; Takara Bio Inc., Japan). The amplified products were electrophoresed on agarose gel, and then visualized under UV transilluminator at 320 nm. Nasal swab and lung homogenate samples were classified as nPCR-positive or -negative.

Statistical analysis: Statistical analysis was conducted using Pearson's correlation coefficient.

RESULTS

Detection of *M. hyopneumoniae* in experimentally infected pigs: Culture and nPCR analysis were compared for the detection of *M. hyopneumoniae* in the nasal swabs collected at weekly intervals from 6 experimentally inoculated

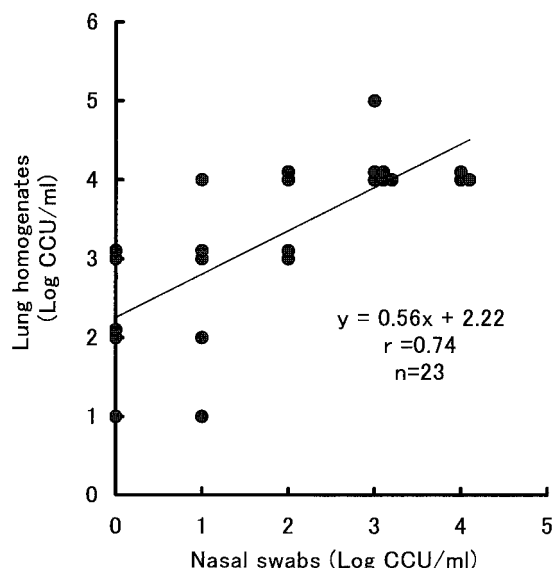


Fig 1. Correlation of viable *M. hyopneumoniae* titers between nasal swabs and lung homogenates collected from 23 necropsied pigs with experimental inoculation. r: Correlation coefficient. n: Number of pigs tested.

pigs. Nasal swabs prior to inoculation were negative for *M. hyopneumoniae* in all pigs, whether the culture method or nPCR was used. Thereafter, *M. hyopneumoniae* was isolated from the nasal swabs of one pig (16.7%) at 2 weeks PI, and 3 pigs (50%) at 3 weeks PI by the culture method, whereas detection by nPCR yielded 83.3% (5/6), 33.3% (2/6), and 66.7% (4/6) for 1 to 3 weeks PI, respectively. At 4 weeks PI, all pigs were positive for *M. hyopneumoniae* in nasal swabs by both methods (Table 1).

Figure 1 shows the correlation of the titers of viable *M. hyopneumoniae* between lung homogenates and nasal swabs collected from 23 of the necropsied pigs with experimental inoculation. The titers of organisms in the nasal swabs were significantly correlated to those in the lung homogenates ($P < 0.01$). Linear regression analysis of these data showed regression coefficients of 0.74. However, the titers of viable organisms in the majority of nasal swabs were generally about one-hundredth of those in the lung homogenates.

The results of culture and nPCR of lung homogenates and nasal swab samples for the detection of *M. hyopneumoniae* from the 23 pigs that underwent experimental inoculation are shown in Table 2. All animals had developed lung

Table 2. Detection of *M. hyopneumoniae* by culture and nPCR in nasal swabs and lung homogenates collected from 23 experimentally inoculated pigs at necropsy

		No. of pigs positive (%)	No. of pigs with positive nasal swabs (%)	
			Culture	nPCR
Lung homogenates	Culture	23 (100)	18 (78.3)	20 (87.0)
	nPCR	22 (95.3)	18 (78.3)	19 (82.6)

lesions typical of MPS by necropsy. *M. hyopneumoniae* was isolated from all of the lung homogenates from the 23 necropsied pigs by the culture method. *M. hyopneumoniae* was isolated from both lung homogenates and nasal swabs from 18 (78.3%) pigs, and organisms were also isolated in 5 lung homogenates from pigs with culture-negative nasal swabs. The nPCR detected *M. hyopneumoniae* in 22 (95.7%) lung homogenates and 20 (87.0%) nasal swabs, with both samples being positive in 19 (82.6%) pigs; 3 samples were detected only in the lung homogenate and 1 only in the nasal swab.

Detection of *M. hyopneumoniae* in clinical samples: The results of detection of *M. hyopneumoniae* in pigs obtained by culture with lung homogenates and nPCR with nasal swabs are summarized in Table 3. *M. hyopneumoniae* was detected in nasal swabs of 9 (25.0%) of the 36 pigs by nPCR, but was detected in the lung homogenates of 20 (55.6%) pigs. The results of lung homogenate culture and nasal swab nPCR were both positive for *M. hyopneumoniae* in 6 (16.7%) pigs from which more than 10^3 CCU/ml of *M. hyopneumoniae* was isolated from lung lesions. Lung homogenate culture enabled the detection of 14 (38.9%) pigs positive for *M. hyopneumoniae* that were undetected by nPCR with nasal swabs, while nPCR with nasal swabs detected *M. hyopneumoniae* in 3 (8.3%) pigs for which lung homogenate culture gave negative results. The remaining 13 pigs showed negative results in both lung homogenates and nasal swabs.

The results of nPCR with nasal swabs and lung lesion development are summarized in Table 4. A total of 18 (50%) of 36 pigs had the typical lung lesions of MPS at slaughter. Using the culture method, *M. hyopneumoniae* was isolated from the lung homogenates of 12 (75.0%) of the 16 pigs that developed mild to moderate lung lesions (0.1–5.0%), and from 1 (50.0%) of the 2 pigs that developed severe lung lesions (5.1–20.0%). Using nPCR with nasal swabs, *M. hyopneumoniae* was detected in the nasal swabs of 5 (31.3%) of the 16 pigs with mild to moderate lung lesions, and in 1 (50.0%) of the 2 with severe lung lesions.

DISCUSSION

To evaluate the suitability of nasal swabs as a potential diagnostic sample for *M. hyopneumoniae* infection in the pig, the lung homogenates and nasal swab samples from experimentally inoculated pigs were examined for *M. hyopneumoniae*. In a longitudinal study, *M. hyopneumoniae* was commonly detected in the nasal swabs of pigs between 2 and

Table 3. Detection of *M. hyopneumoniae* in lung homogenates and in nasal swabs collected from naturally infected pigs

Lung homogenates (logCCU/ml)	Detection of <i>M. hyopneumoniae</i> by nPCR in nasal swabs		Total
	No. of pigs positive	No. of pigs negative	
Negative	3	13	16
3	1	2	3
4	0	6	6
5	2	2	4
≥6	3	4	7
Total	9	27	36

Table 4. Comparison of *M. hyopneumoniae* detection rates in lung homogenates and nasal swabs from naturally infected pigs with lung lesions

Lung lesions (%)	No. of pigs	No. of pigs positive for <i>M. hyopneumoniae</i> (%)	
		Culture with lung homogenates	nPCR with nasal swabs
0.0	18	7 (38.8)	3 (16.7)
0.1–5.0	16	12 (75.0)	5 (31.3)
5.1–20.0	2	1 (50.0)	1 (50.0)
Total	36	20 (55.6)	9 (25.0)

4 weeks PI by the nPCR and culture method. *M. hyopneumoniae* was detected in the nasal swabs of pigs as early as 1 week PI by nPCR, but was not isolated by culture. Since inflammatory cell infiltration into the airways and T cell accumulation around the bronchi would start at 2 to 3 weeks PI [2], *M. hyopneumoniae* may be transitorily present in small quantities in the nasal cavity. In addition, *M. hyopneumoniae* may have originated from the inoculation materials and remained in the nasal cavity for a week after inoculation. Because *M. hyopneumoniae* inoculated into the nasal cavity would be reduced in number or be degraded there, this may have contributed to the failure to isolate *M. hyopneumoniae* from the nasal cavity of the pigs by the culture method at 1 week PI. We also examined nasal swab and lung homogenate samples from necropsied pigs for the titration of viable *M. hyopneumoniae* by the culture method. The present study found that the titer of viable organisms in nasal swabs and lung homogenates was significantly correlated ($P < 0.01$) in the experimentally inoculated pigs, although those in the nasal swabs were one-hundredth of

those in lung homogenates. It is known that *M. hyopneumoniae* colonizes the surface of the ciliated epithelium of the trachea, bronchi, and bronchioles of pigs [4]. Furthermore, although *M. hyopneumoniae* does not normally colonize the nasal cavity of pigs, it was isolated from the nasal swabs of the affected pigs [9]. This reduction may have occurred through an increase of organisms in the bronchi and alveoli of the infected pigs via the upper respiratory tract. *M. hyopneumoniae* originating from the lung lesions may have been present in the nasal cavity of the infected pigs and may have been detected by nPCR. A previous study described that the presence of *M. hyopneumoniae* is evidenced during the whole course of the disease, while clinical and pathological signs, depending on the stage of the disease, must be taken into account [7]. Thus, the nasal swab samples may be suitable for nPCR under experimental conditions.

In the naturally infected pigs, 36 pairs of both nasal swab and lung homogenate samples from individual pigs were tested. By contrasting the results of nPCR using nasal swabs with those of culture of lung homogenates, discrepancies in detection between the two sampling methods were observed in 17 of 36 pair samples. The presence of gross lung lesions is relatively well correlated with the results of detection of organisms from lung homogenates and nasal swabs. A recent study compared bronchial swabs with nPCR and lung lesions, and concluded that nPCR correlated well with histopathological lesions [7]. As increases in the number of organisms in the lung develop the pneumonic lesions, *M. hyopneumoniae* may be more easily found in nasal swabs of pigs with lung lesions. However, it can be seen that the detection rate in the nasal swabs tested by nPCR was considerably lower than that in lung homogenates. Even nPCR failed to detect organism in nasal samples of the pigs with lung lesions or large numbers of organisms in the lung. DNA amplification, including PCR primers as an internal control, can clarify the presence of inhibitors for PCR in the nasal samples [11]. Thus, diagnosis of MPS by nPCR with a nasal swab on individual pigs is not suitable under field conditions.

Because of its high sensitivity, even airborne *M. hyopneumoniae* can be detected by nPCR under experimental and field conditions [18]. Though several investigations using PCR techniques have been described to detect *M. hyopneumoniae* from nasal swabs [6, 8, 16], Kurth *et al.* [11] indicated that nasal swabs were not reliable samples for detection of *M. hyopneumoniae*. Our results indicated that diagnosis of MPS by nPCR with a nasal swab on individual pigs is suitable under experimental conditions. As the case stands now, nPCR with nasal swabs should be available only for monitoring disease status at the herd level.

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