Nested PCR for Detection and Typing of Porcine Reproductive and Respiratory Syndrome (PRRS) Virus in Pigs

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ABSTRACT. A nested polymerase chain reaction (PCR) was developed to detect porcine reproductive and respiratory syndrome (PRRS) virus. A common primer set for European and North American type isolates of PRRS virus was designed for reverse transcription PCR, and a specific primer set for each of the 2 type isolates was designed for nested PCR. The PCR that used a specific primer set detected the corresponding type of the virus at a level equivalent to 1 TCID₅₀/100 µl, but not the other type of isolates. Therefore, the method clearly differentiated the 2 types of virus from each other. The detection of PRRS virus by the nested PCR was as sensitive as virus isolation in cultures of porcine alveolar macrophages from infected pigs at the acute stages, and was more sensitive from pigs at the convalescent stages. The infecting virus type was determined by use of 2 specific primer sets even when virus isolation was negative in naturally infected pigs. It was concluded that the nested PCR is useful for diagnosis and typing of PRRS virus and studies of persistent infection by the virus. — KEY WORDS: diagnosis, PCR, PRRS virus, sensitivity, virus typing.

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The causative agents of a viral disease of pigs characterized by reproductive and respiratory syndrome (PRRS) were isolated in Europe [7, 22], North America [5, 6] and also in Japan [9, 15, 17]. The virus, named PRRS virus, is a member of the Arteriviruses [14] and consists of two serotypes called European and North American types [21]. Sequence analysis revealed a high degree of genetic variation between the isolates of the 2 types [10, 11, 13, 14]. Japanese isolates had identical serological properties and a nucleotide sequence closely related to the North American isolates [15, 16]. PRRS virus propagated persistently for several weeks in pigs [4, 17, 18, 23] and reexcretion of the virus after long latency was demonstrated by contact infection experiments [1]. These data indicated the necessity of studies on persistence of the virus in animals. The isolation and titration of PRRS virus have been carried out with porcine alveolar macrophage (PAM), MARC-145 and CL2621 cell cultures [2, 3, 8, 22]. PAM cultures are highly sensitive to the virus but the application of the cells is hampered by the necessity of frequent collection of the cells from euthanized specific pathogen-free (SPF) pigs. Therefore a continuous cell line, MARK-145 cells, was thought to be more suitable for the study of PRRS virus than PAM, but it was not always suitable for virus isolation since several isolates were unable to grow in the cells [3, 8]. Thereafter, a polymerase chain reaction (PCR) that detected the virus at levels of 10–30 TCID₅₀/ml was developed [12, 19, 20]. However, detection of the virus by the PCR from infected pigs was less sensitive than the virus isolation in PAM cultures [12, 19]. This study was conducted to assess the nested PCR for detection of the virus (or viral RNA) in experimentally and naturally infected pigs. The results revealed that the method was more sensitive than virus isolation especially in chronically infected pigs and could differentiate between European and North American serotypes at the RNA level.

MATERIALS AND METHODS

Viruses: Two Japanese isolates, EDRD-1 (EDRD) [15] and Chiba92-1 (Chiba) [17], 1 US isolate, 46448 (US) [15], and 1 European isolate, Lelystad virus (LV) [22] were used. Titration of the virus in the serum or tissues were carried out in porcine alveolar macrophage (PAM) cultures soon after the sampling, in general. On the other hand, the samples were studied by the PCR after storage at −80°C for 2.5–4 years.

Experimental clinical samples: Two groups of pigs infected experimentally were used. One group comprised 3 of those described previously [17]. Briefly, 5-day-old hysterectomy-produced and colostrum-deprived pigs were inoculated intranasally with 10⁶ TCID₅₀ of the Chiba strain. The pigs were examined 7, 14 and 42 days after inoculation. In the second group, 2 60-day-old SPF pigs were inoculated with the virus as described above and serum samples were collected weekly.

Field clinical samples: Two groups of samples obtained from different sources were used. The first group was from pigs showing signs of PRRS, and the second group was from pigs that died of acute Escherichia coli infection. The
samples were tested by the PCR since typical changes of PRRS such as interstitial pneumonia were found in the lung by postmortem examinations.

RNA samples: Viral genomic RNA was extracted with a commercial RNA isolation kit (RNAzol B, TEL-TEST Inc., Tx, U.S.A.). Virus infected cell culture fluid, serum diluted with an equal volume of phosphate buffered saline or the supernatant of a 10% homogenate of tissues, in an amount of 0.2 ml was mixed with 0.8 ml of the RNAzol B. The mixture was then treated according to the manufacturer's manual. The final precipitate of RNA was suspended in 20 μl of RNase-free distilled water, and used for reverse transcription (RT)-PCR.

Primers: The designs of primers for RT-PCR amplification and nested PCR were based on the nucleotide sequences of open reading frames (ORF) 6 and 7 of the EDRD [10] and the LV [14] strains. First, a common primer set, Nos. 21 and 26 (Pr21/26) that can amplify both EDRD RNA and LV RNA was designed. Then, specific primer sets, Pr22/24 and Pr23/25, that probably amplify only EDRD RNA and LV RNA, respectively, were designed inside of the amplification site of Pr21/26. The primer locations and sizes of the predicted amplified products are shown in Fig. 1. Their sequences (5' to 3') are as follows:

- No. 21, GTACATTCGCCCCCTGCC;
- No. 22, TCGTTTCGCCGTCCCCGCTC;
- No. 23, CGCCTGTGAGAAACCCCGGAC;
- No. 24, TTGACGAGCAAGACACATTGC;
- No. 25, TCGATTTCAGACGAGGGAG;
- No. 26, GCCCTAATGTGAATAGGTGAC.

The oligonucleotide primers were used at a concentration of 20 pmol per μl for the RT-PCR and nested PCR.

RT-PCR: The step of RT was performed as follows: The mixture of 1 μl of sample RNA, 1 μl of a lower primer (No. 26) and 18 μl of the reagent mixture of a Takara RNA PCR Kit (Takara Shuzo Co., Shiga, Japan) prepared according to the instructions of the supplier was incubated at 42°C for 25 min; at 99°C for 5 min, and at 55°C for 1 sec. Then it was mixed with 1 μl of an upper primer (No. 21) and 79 μl of the reagent mixture of the kit, prepared as described in the instructions. The mixture was subjected to 30 amplification cycles, each cycle consisting of 30-sec denaturation, 30-sec annealing and 1.5-min elongation steps at temperatures of 94, 50, and 72°C, respectively. The PCR was ended with a final elongation step of 3 min at 72°C.

Nested PCR: The nested PCR was carried out in a 22 μl reaction mixture containing 1 μl of the RT-PCR product, 1 μl each of the upper (No. 22 or 23) and lower (No. 24 or 25) primers and 19 μl of reagent mixture provided by the Takara PCR Kit. The PCR was performed by 20 amplification cycles, each cycle consisting of 1-min denaturation, 1-min annealing and 2-min elongation steps at 94, 55, and 72°C, respectively. The PCR was ended with a final elongation step of 10 min at 72°C. The amplified products were detected by electrophoresing 10 μl samples through a 2% agarose gel in TBE buffer (45 mM Tris-borate, 1 mM EDTA (pH 8.0)).

Restriction enzyme digest: One-half μl of the nested PCR products purified by phenol extraction and ethanol precipitation was digested in a 10 μl mixture containing restriction enzymes PvuII (7.5 units) and Rsal (8.5 units), respectively, at 37°C for 90 min. The digests were analyzed by electrophoresis as described above.

RESULTS

Specificity and sensitivity of the PCR: The RT-PCR using Pr21/26 amplified the expected sizes of the products, 668 bp for EDRD, Chiba and US, and 645 bp for LV (Fig. 2). By the nested PCR, part (347 bp) of the cDNA of EDRD, Chiba, and US was amplified only by using Pr22/24, not Pr23/25. In contrast, cDNA of LV was amplified markedly by use of Pr23/25, and, unexpectedly, also very faintly by using Pr22/24 (not visible in the printed photograph) (Fig. 2).

The products of the nested PCR were treated with restriction enzymes PvuII and Rsal, respectively, to confirm the presence of the restriction enzyme cleavage sites shown in Fig. 1. As shown in Fig. 3, PvuII cleaved the products of
EDRD, Chiba and US into the predicted sizes of 2 fragments, 172 and 177 bps, although the fragments were detected as a single band because of their very close sizes.

The enzyme failed to digest the product of LV, as expected. The other enzyme, Rsal, cleaved only the product of LV into 3 fragments of the predicted sizes of 199, 110 and 45 bps. The third smallest fragment is barely seen in the figure.

The sensitivity of PCRs for detecting PRRS virus was tested with RNA samples extracted from cell culture fluid infected with EDRD by 2 different processes as follow: First, viral RNA was extracted from each of a series of 10-fold dilutions of the culture fluid. Second, viral RNA extracted from the undiluted culture fluid was diluted 10-fold serially in RNaase-free distilled water. The RT-PCR using Pr21/26 generated a visible product at dilutions of the 2 RNA samples corresponding to about $10^5$ TCID$_{50}$/100 µl (Fig. 4, RT-PCR). When the PCR product was reamplified by the nested PCR with Pr22/24, the expected size of the product was detected at a dilution corresponding to 1 TCID$_{50}$/100 µl (Fig. 4, Nested PCR). Thus, the amount of viral RNA in a sample could be titrated equally well by either of the 2 methods. The second method was used in the following tests.

Comparison of detection of PRRS virus by virus isolation and PCR: As shown in Table 1, amplification of viral RNA in the tissues of pigs was observed by the RT-PCR and the nested PCR with Pr22/24, but not by the nested PCR with Pr23/25. In pigs sacrificed 7 and 14 days after inoculation, the reciprocal of the dilution of the sample RNA that amplified a specific product by the nested PCR roughly coincided with the infectivity of the respective tissues. In a pig sacrificed 42 days after inoculation, infectious virus was recovered only in the tonsil out of 7 tissues tested. In contrast, by the nested PCR, amplification of viral RNA was detected in 5 tissue samples (Table 1 and Fig. 5). In an additional experiment, as shown in Table 2, detection of virus in sera collected weekly from 2 pigs by virus isolation and the nested PCR again coincided in early weeks after inoculation. It was noticed that the nested PCR detected the virus in a serum sample collected 5 weeks after inoculation that contained serum neutralizing antibody and failed to show infectivity.

As shown in Table 3, the PCR was applied to field samples. In the first group, PRRS virus was isolated from 3 sera in PAM cultures after the primary inoculation, and from 4 lung samples after the first subinoculation. No virus was isolated from a lung sample after the subinoculation. Interestingly, large amounts of the product with the size of PRRS viral RNA were visualized by using RNA extracted from the 5 lung homogenates including a virus-isolation negative sample, but did not by using RNA from 3 sera, by means of the RT-PCR. However, all products of the RT-PCR amplified a product having a molecular size of PRRS virus by the nested PCR with Pr22/24, but not by Pr23/25. In the second group, the virus was not isolated from any of the 11 lung samples by 2 serial subinoculations in MARC-145.

![Figure 3](image-url) Digestion of the nested PCR products with restriction enzymes PvulII and Rsal.

![Figure 4](image-url) Sensitivity of the RT-PCR and nested-PCR in detecting PRRS virus. Products of RT-PCR were reamplified by the nested PCR using a specific primer set. A: RNA extracted from serial 10-fold dilutions of a virus-infected culture fluid was used. B: RNA extracted from the undiluted fluid and then diluted serially was used. The TCID$_{50}$ corresponding to the dilution is shown. M: marker; C: control culture fluid; W: distilled water.

![Figure 5](image-url) Detection of PRRS virus in tissues of pigs 42 days after inoculation. 1: lung; 2: tonsil; 3: pulmonary lymph node; 4: mesenteric lymph node; 5: liver; 6: spleen; 7: serum; M: marker.
Table 1. Comparison of virus isolation and detection of virus by 
PCR in pigs inoculated with the Chiba strain of PRRS virus, in 
acute and convalescent stages

<table>
<thead>
<tr>
<th>Days after</th>
<th>Tissues tested</th>
<th>Virus infectivity</th>
<th>RT-PCR Pr21/26</th>
<th>Pr22/24</th>
<th>Pr23/25</th>
</tr>
</thead>
<tbody>
<tr>
<td>inoc. 7</td>
<td>Lung 2.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>$10^0$&lt;sup&gt;b&lt;/sup&gt;</td>
<td>$10^1$&lt;sup&gt;b&lt;/sup&gt;</td>
<td>$10^3$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Tonsil 2.8</td>
<td>$10^2$</td>
<td>$10^3$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Spleen 3.0</td>
<td>$10^3$</td>
<td>$10^3$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Lymph nodes</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>mesenteric</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>pulmonary</td>
<td>1.3</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Serum 5.0</td>
<td>$10^2$</td>
<td>$10^4$</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

| Days 14   | Lung 3.8       | +                 | +              | -        | -       |
|           | Tonsil 3.5     | +                 | +              | -        | -       |
|           | Spleen 5.0     | +                 | +              | -        | -       |

| Days 42   | Lung Neg       | -                 | -              | -        | -       |
|           | Tonsil 2.5     | $10^1$            | $10^2$         | -        | -       |
|           | Spleen Neg     | -                 | +              | -        | -       |
|           | Lymph nodes    | -                 | +              | -        | -       |
|           | mesenteric     | Neg               | -              | +        | -       |
|           | pulmonary      | Neg               | -              | +        | -       |
|           | Serum Neg      | -                 | -              | -        | -       |

Notes:

- a) Log TCID<sub>50</sub>/100 µl of 10% tissue homogenates in porcine macrophage cultures.
- b) Expressed as reciprocal of the dilution of RNA extracted from 20 µl of 10% tissue homogenate that showed positive amplification of the expected size of the products.
- c) Positive (+) or negative (−) amplification. Titration was not carried out.

Table 2. Virus isolation and detection of the virus by PCR from sera of pigs inoculated with the Chiba strain of PRRS virus

<table>
<thead>
<tr>
<th>Pig No. 5</th>
<th>Weeks after inoc.</th>
<th>Pig No. 6</th>
<th>Weeks after inoc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 2 3 4 5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1 2 3 4 5&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Virus isolation&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+ + − + − + + + − −</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RT-PCR&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+ + − − − + + + + +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nested PCR</td>
<td>+ + + + + + + + + +</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Notes:

- a) Neutralizing antibody was detected.
- b) 100 µl of serum was inoculated into the porcine macrophage culture.
- c) RNA extracted from 10 µl of serum was used.

Table 3. Comparison of isolation of PRRS virus and detection of the virus by means of PCR from field cases

<table>
<thead>
<tr>
<th>Sample</th>
<th>Case No. and materials</th>
<th>Virus isolation</th>
<th>RT-PCR Pr21/26&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Pr22/24</th>
<th>Pr23/25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>07 Serum +&lt;sup&gt;b&lt;/sup&gt;</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>09 Serum +</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>12 Serum −</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>07 Lung (+)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>08 Lung (+)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>10 Lung −</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>11 Lung (+)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>12 Lung (+)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

Notes:

- a) Primers used. +, −: positive or negative amplification.
- b) Results in porcine alveolar macrophages. +: positive after the primary inoculation, (+): positive after the first subinoculation, −: negative after the first subinoculation.
- c) 11 samples of the group showed same results.
- d) Results of 2 serial subinoculations in MARC-145 cell cultures.

cell cultures. Although virus was not detected by the RT-PCR, it was detected in all samples by the nested PCR with Pr22/24, but not by the PCR with Pr23/25. Thus, it was shown that pigs in the 2 groups had been infected with PRRS virus of the North American type.

DISCUSSION

In the study using the virus-infected cell culture fluid, the specificity of the RT-PCR and the nested PCR was proven by the sizes of the amplified products, restriction enzyme analysis of the products, and by a set of various controls (data not shown). The sensitivity of the nested PCR for detection of PRRS virus was 100 times greater than that of the RT-PCR, and the nested PCR detected the virus at a level of 1 TCID<sub>50</sub>/100 µl. The combination of the RT-PCR and the nested PCR that used a common primer set and 2 specific primer sets, respectively, could apparently distinguish between the European type and the North American type viruses, which are different from each other serologically [14, 19]. This combination made it possible to diagnose PRRS virus infection quickly and simultaneously to determine the type of the infecting virus, even when virus isolation was negative.

The nested PCR could detect the virus (or viral RNA) in the tissue of experimentally infected pigs during the acute or subacute stages as sensitively as the infectivity tests in PAM cultures, and the virus was detected more efficiently than in the cultures during convalescent stages, in contrast to previous results [12, 19, 20]. Thus, the procedure is not only an alternative method of virus isolation, but it should be very useful for detection of the virus and viral RNA which might be present in a noninfectious form during the persistent infection.

Similar to the experimental cases, detection of the virus by the nested PCR was more sensitive in field samples than by the virus isolation test. The comparative sensitivity of the 2 methods differed from sample to sample as shown in Table 3. In the first group of field samples, the virus was isolated readily from serum specimens and later from the lung samples (Table 3). In contrast, by the RT-PCR, amplification of viral cDNA was not visualized in the serum
specimens but was clearly seen when lung samples were tested. The results suggest that serum contained only newly produced infectious virus because of successive clearance of the virus from the blood stream, and the lung contained a large accumulation of incomplete or inactivated viruses. In the second group of field samples, the virus could not be isolated in MARC-145 cell cultures but was demonstrated by the nested PCR in all samples which had been kept for more than 4 years at $-80^\circ\text{C}$. It is not known whether the virus was inactivated during storage or whether the cells used for virus isolation were not suitable for the virus. Thus, virus isolation must be largely influenced by such factors as timing of collection of samples from affected pigs, condition of maintenance of the samples, or the cell type used for the isolation. In contrast, the PCR was superior to virus isolation in sensitivity, as it was not influenced by those factors. However, it is necessary to investigate the applicability of the test with various isolates, since the PRRS viruses have nucleotide sequences of relatively high diversity even among North American isolates [11].

REFERENCES


