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## A Rapid and Highly Sensitive Method for Diagnosis of Equine Influenza by Antigen Detection Using Immuno-PCR

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### Abstract

A rapid and highly sensitive method for diagnosis of influenza by detecting viral antigens using immuno-PCR has been developed. The sensitivity of the immuno-PCR for the detection of the nonstructural protein 1 (NS 1) antigenically common among influenza A viruses was  $10^{1.0-2.0}$  times higher than that of RT-PCR for the detection of the viral genome. For the detection of the hemagglutinin (HA) subtype-specific antigen, this assay using anti-HA monoclonal antibodies attained a sensitivity of up to  $10^{7.0-8.0}$  times higher than those by virus isolation or by RT-PCR.

### Introduction

Spread of equine influenza is mainly due to transportation of infected horses. Since race horses are now frequently transported by airplanes over the world, possibility of the international transmission of infectious diseases to horses and related animal species becomes higher. Diagnosis of viral infections has been made by isolation of the causative virus or by detecting antibodies specific to the virus. Virus isolation can be made only from animals of acute phase of infection and takes for several days. Serological diagnosis can be

made after the acute phase of infection when detectable are produced. A more rapid and sensitive method, therefore, has been demanded for diagnosis of equine influenza at the quarantine service.

Sano *et al.* (1992)<sup>14)</sup> developed a highly sensitive antigen detection system that combines enzyme-linked immunosorbent assay and polymerase chain reaction (Immuno-PCR). Immuno-PCR-based system for the diagnosis of bovine herpesvirus 1 infection has been described<sup>9)</sup>. In the present study, we developed a highly sensitive immuno-PCR assay for the detection of the nonstructural protein

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1 (NS 1) that is known to be antigenically common among influenza A viruses<sup>2)</sup> and for the detection of the hemagglutinin (HA) antigen using monoclonal antibodies to the H3 HA of equine influenza virus. The sensitivity and specificity of the assay were compared with those of virus isolation and the viral genome detection by RT-PCR from the samples of experimentally infected animals.

### Materials and methods

#### Viruses

Virus strains used in the present study are A/Aichi/2/68 (H3N2) (Aichi/68), and A/equine/La Plata/1/93 (H3N8) (Eq/LP/93). Viruses were propagated in 11-day-old embryonated chicken eggs at 35°C for 48hr<sup>6)</sup>. Viral infectivities were titrated by plaque assay in Madin-Darby canine kidney (MDCK) cells.

#### Cloning of the NS 1 gene

Viral RNA of Eq/Miami/63 was extracted by the method of Bean *et al.* (1980)<sup>1)</sup>, and used for reverse transcription with the random oligonucleotide hexamer<sup>4)</sup>. PCR<sup>12)</sup> was performed using oligonucleotide primers 5'-GCAAAGCAGGGTGACAAAAC-3' (NSF 2) and 5'-GAAACAAGGGTGTTTTATC-3' (NSR 866) with slightly modification of the conditions of amplification consisting of 35 cycles of 0.5min at 95°C, 1min at 50°C, and 3 min at 72°C. The PCR product was purified by Gene Clean Kit (Bio 101) and cloned into pCR II plasmid (Invitrogen) according to the manufacturer's instructions. And then, the cDNA of the NS 1 gene was amplified using oligonucleotide primer 5'-ACAGAATTCTAATGGATTCCAACAC-3' (NS24-*EcoRI*) and the SP 6 primer. The product was digested with *EcoRI* and ligated into *EcoRI*-cleaved pPRO EX-1 (GIBCO BRL). The constructed plasmid was transformed into *E. coli* JM 109

strain.

#### Expression of the NS1

Recombinant bacteria were cultured overnight at 37°C in 5 ml LB medium, supplemented with 100µg/ml ampicillin, and then the culture was added to 1,000ml LB medium containing ampicillin (100µg/ml). After 2 hr in an orbital shaker at 37°C, 0.6mM isopropyl-thiogalacto-pyranoside (Takara) was added to induce the NS 1 production, and incubated for 5 hr at 25°C. The expressed recombinant NS 1 was purified according to the expression kit protocol (GIBCO BRL).

#### Monoclonal antibodies to the NS1 and the hemagglutinin of A/equine/Miami/1/63(H3N8) virus

Monoclonal antibodies to the NS 1 (NS 13/3) and to the hemagglutinin (HA) of A/equine/Miami/1/63 (H3N8) virus (M 9/2 and M41/1) were prepared according to Kida *et al.* (1982).

#### Experimental infection of mice

Aichi/68 virus of 10<sup>4.0</sup> plaque forming units (pfu) in 50µl was intranasally inoculated into 5-week-old BALB/c mice (Nippon SLC). Mice were sacrificed every 12hr from 1 day until 4 days post inoculation (pi), 7, 14, 28, 42, and 56 days for the sampling of the nasal and lung washes. These washes were collected in 0.5ml of PBS containing 0.1% bovine serum albumin (BSA) and assayed<sup>16)</sup>.

#### Experimental infection of a horse with A/equine/La Plata/1/93(H3N8) virus

Experimental infection of horse with the allantoic fluid of chicken embryos infected with Eq/LP/93 influenza virus (1:320 hemagglutinating units) was carried out in a BL-3 containment. Virus was inoculated into the nasal cavities using a nebulizer. Nasal swabs were collected in 1 ml of PBS containing 0.1% BSA every day until 10 days pi.

Animal experimentations were performed according to the Guideline for Animal Experimentation at Hokkaido University Graduate School of Veterinary Medicine.

#### *Virus isolation*

Virus isolation and infectivity titration were made by plaque formation on MDCK cells. Serial dilution of the samples were inoculated on MDCK cell monolayers. After 1 hr adsorption, the inoculum was removed and the cells were overlaid with Eagle's minimal essential medium (EMEM) containing 1 % Bacto-Agar (Difco) and 5 µg/ml trypsin (Difco). After incubation at 35°C for 2 days in the 5 % CO<sub>2</sub> atmosphere, cells were overlaid with EMEM containing 1 % Bacto-Agar and 0.005% neutral red, followed by plaque count.

#### *Virus genome detection*

Viral RNA was extracted from samples of animals according to the method by Bean *et al.*, (1980)<sup>11</sup>. cDNA was synthesized by using reverse transcriptase (Gibco BRL) and random 6-base synthetic primer complementary to the 3' terminus of the viral RNA. Polymerase chain reaction (PCR) of the partial NP and NS genes was performed according to Saiki *et al.* (1985)<sup>12</sup>, using the following synthetic primers; NSF 2 : 5'-GCAAAAGCAGGGTGACAAAAC-3', NSR866 : 5'-GAAACAAGGGTGTTCATC-3', NP1141F : 5'-GCTTCAAATGAGAACATGGA-3', and NP1501R : 5'-TTGTCTCCGAAGAAATAAGA-3'.

#### *Immuno-PCR*

For antigen detection, the allantoic fluids of chicken embryos infected with Aichi/68 or Eq/La Plata/93 virus, and the samples from experimentally infected animals were centrifuged at 2,000rpm for 10min and serial 10-fold dilutions of them were prepared in PBS. Each dilution was put into 10 times volume of dis-

ruption buffer (0.05M Tris-HCl [pH7.8], 0.5% Triton-X-100, 0.6M KCl). Each well of 96-well plates (Falcon3911; Becton Dickison) was coated with 50µl of each dilution at 4 °C overnight. The plates were blocked with 1 % BSA in phosphate buffered saline (PBS). Fiftyµl of mixture of 5 monoclonal antibodies to the HA of Aichi/68 or Eq/LP/93 viruses, or monoclonal antibody to the NS 1 of A/equine/Miami/1/63 (H3N8) were diluted 1 : 5,000 in PBS containing 0.5% BSA and 0.05% Tween 20 (BSA 5 T), and added to each well of the plates. After the adsorption of the monoclonal antibodies, 1 : 2,000 dilution in BSA 5 T of biotin-labeled goat antibodies to mouse immunoglobulin G Fc (EY Laboratories, Inc.) were added. The procedure of immuno-PCR was performed according to Mweene *et al.* (1995)<sup>9</sup>, with slight modifications. The *Hind*III-*Acc*I fragment of plasmid DNA, pUC19<sup>18</sup>, was selected for amplification marker. Biotin was incorporated at 3' terminus by a filling-in reaction with Klenow enzyme (Takara) in the presence of biotin-14-dATP (Gibco BRL). Free nucleotides were removed by using Sephadex G-50 (Boehringer Mannheim). Synthetic oligonucleotides PC 1 : 1070 (5'-GCTGTAGGTATCTCAGTTCGGTGTAGGTCG-3') and PC 2 : 1261 (5'-CACCGCCTACATACCTCGCTCTGCTAATCC-3') of the ori gene sequence of pUC19 were used as primers. The ELISA part of immuno-PCR was performed as described previously (5), except that free streptavidin (Wako) diluted 1 : 3000 (5 ng/µl) with BSA 5 T was added in place of the conjugate and incubated for 1 hr at room temperature. After the wells were washed with PBS containing 0.05% Tween 20 (PBST), 50µl of 1.5x10<sup>-21</sup>mol of the marker DNA in BSA 5 T was added to each well and incubated. The plates were washed 10 times and subjected to PCR<sup>12</sup>. One µl each of PC 1 and PC 2 (15pmol), 4µl of deoxynu-

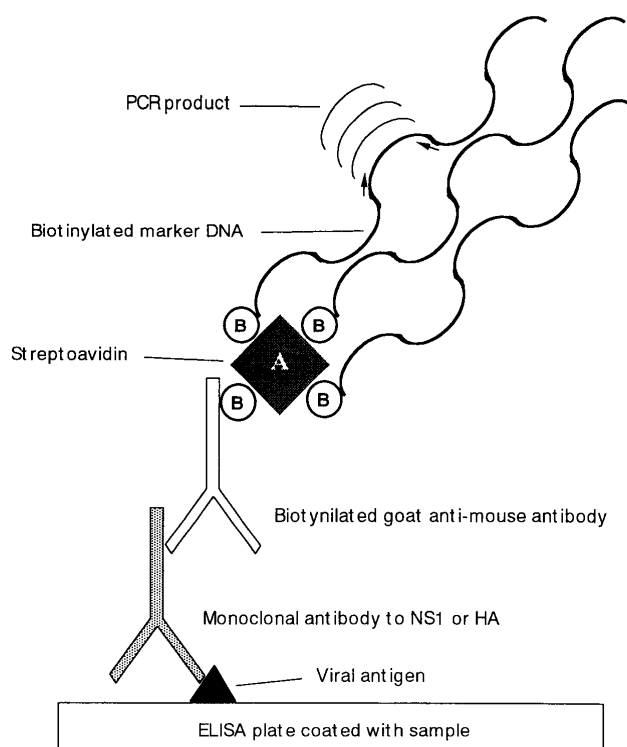


Fig. 1. Schematic representation of the immuno-PCR method employed in this study. A sample was immobilized on ELISA microtiter plate. If viral antigen is present in the sample, antigen-antibody complex is obtained by incubating with a antigen specific monoclonal antibody. Biotinylated anti-mouse IgG antibody is used to build an avidin bridge linking the antigen-antibody complex to a biotinylated marker DNA. Streptoavidin has four biotin binding sites and binds biotin with an higher affinity than antigen-antibody complexes. The amount of antigen present in the sample is then quantified by PCR amplification of the marker DNA.

cleoside triphosphates (10mM), 5 $\mu$ l of 10x reaction buffer (Nippon Gene), and 0.15 $\mu$ l (0.75U) of Gene Taq DNA polymerase (Nippon Gene) were added to each well, mixed, and overlaid with 40 $\mu$ l of mineral oil. Thirty amplification cycles were carried out in a PTC-100programmable thermal controller (MJ Research, Inc.); 95 $^{\circ}$ C, 30sec of denaturation, 58 $^{\circ}$ C, 1min of annealing, and 72 $^{\circ}$ C, 2min of extension, and final extension, 72 $^{\circ}$ C for 3 min (Fig. 1). The product of 191 bp size predicted upon the published gene sequence<sup>18)</sup>,

was visualized by electrophoresis in 2 % agarose gels containing ethidium bromide. No amplification product was detected when one of the following materials was omitted: antigen (virus particles), primary antibody (anti-HA or anti-NS 1 molecule of the viruses), secondary antibody (biotinylated goat anti-mouse), streptoavidin, marker DNA, Taq polymerase, or primers (data not shown).

## Results

To assess the sensitivity of the present immuno-PCR method for the detection of the NS 1 or the HA antigens, serial 10fold dilutions of the allantoic fluids (10<sup>7.9-8.0</sup> PFU/0.1 ml) were tested. As the first antibody, NS13/3 and monoclonal antibody cocktail (A13/1, A110/2, A48/2, M9/2, and M41/1)<sup>7)</sup> were used to detect the NS 1 and HA antigens, respectively. As shown in Table 1, immuno-PCR detected the NS 1 or the HA antigens up to in a dilution of 1 : 10<sup>5.0-6.0</sup> or 1 : 10<sup>11.0-12.0</sup>, respectively. For the detection of virus genomes, the allantoic fluids of the chicken embryos infected with each virus were then tested by RT-PCR. PCR products of NS or NP genes were detected in the allantoic fluids up to a dilution of 1 : 10<sup>4.0</sup>. Representative agarose gel electrophoresis image of immuno-PCR and RT-PCR were shown in Fig. 2. The results of three independent tests showed that immuno-PCR was more sensitive than either plaque-forming assay or RT-PCR.

Results of the detection of the NS 1 antigen by immuno-PCR and the NS gene by RT-PCR from the samples of mice infected with Aichi/68 virus are shown in Fig. 3. Viruses were isolated from the nasal washes 72 hr to 96 hr post inoculation (pi), and the infectivity titers were up to 1.2x10<sup>2.0</sup> pfu/0.1ml. Immuno-PCR and RT-PCR detected virus antigen and genomes in earlier phase of infection more sensitively than virus isolation. Sensitivity of

Table 1. Comparison of the sensitivities of three assays on the allantoic fluids of chicken embryos infected with influenza viruses <sup>a)</sup>

Assay	Virus	Target for detection	Titer <sup>b)</sup>
PFU	Aichi/68 (H3N2)		8.0(7.9, 8.1, 8.0)
	Ed/La Plata/93 (H3N8)		7.8(8.0, 7.7, 7.7)
RT-PCR	Aichi/68 (H3N2)	NP gene	4.0(4.0, 4.0, 4.0)
		NS gene	4.0(4.0, 4.0, 4.0)
	Ed/La Plata/93 (H3N8)	NP gene	4.0(4.0, 4.0, 4.0)
		NS gene	4.0(4.0, 4.0, 4.0)
Immuno-PCR	Aichi/68 (H3N2)	HA antigen	12.0(12.0, 12.0, 12.0)
		NS1 antigen	6.0(6.0, 5.0, 7.0)
	Ed/La Plata/93 (H3N8)	HA antigen	11.0(12.0, 10.0, 11.0)
		NS1 antigen	5.0(6.0, 6.0, 3.0)

<sup>a)</sup>The allantoic fluids were diluted as described in the text.

<sup>b)</sup>Titers are shown as log<sub>10</sub>reciprocals of the highest dilution that was positive. Numbers in parentheses are the results of three independent assays.

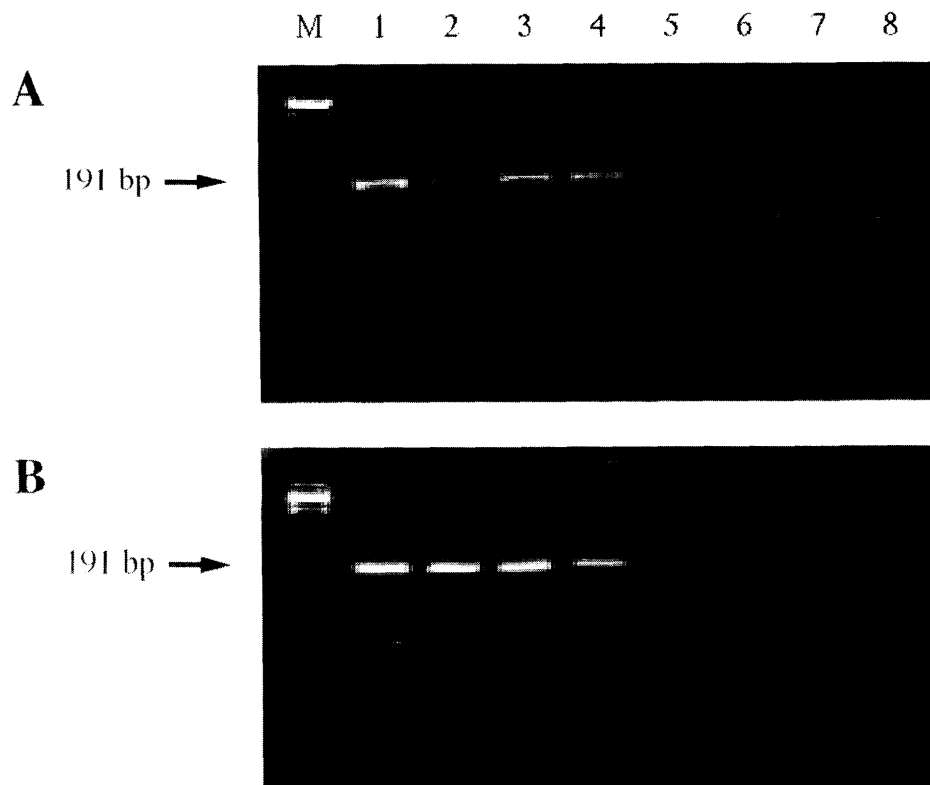


Fig. 2. Sensitivity of immuno-PCR and RT-PCR on the allantoic fluid of embryonated chicken eggs infected with A/Aichi/2/68 (H3N2). (A) Detection of NS1 antigen was performed by immuno-PCR. Lanes 1 through 7, 50  $\mu$ l of 10-fold serial dilutions of antigen starting with  $10^{-1.0}$  up to  $10^{-7.0}$ , respectively. No product was visible from the antigen-negative well (lane 8). (B) Detection of NS gene was performed by RT-PCR. Lanes 1 through 7, 50  $\mu$ l of 10-fold serial dilutions of allantoic fluids starting with  $10^{-1.0}$  up to  $10^{-7.0}$ , respectively. Lane M shows the migration of marker fragments of *Hind* III-digested lambda DNA.

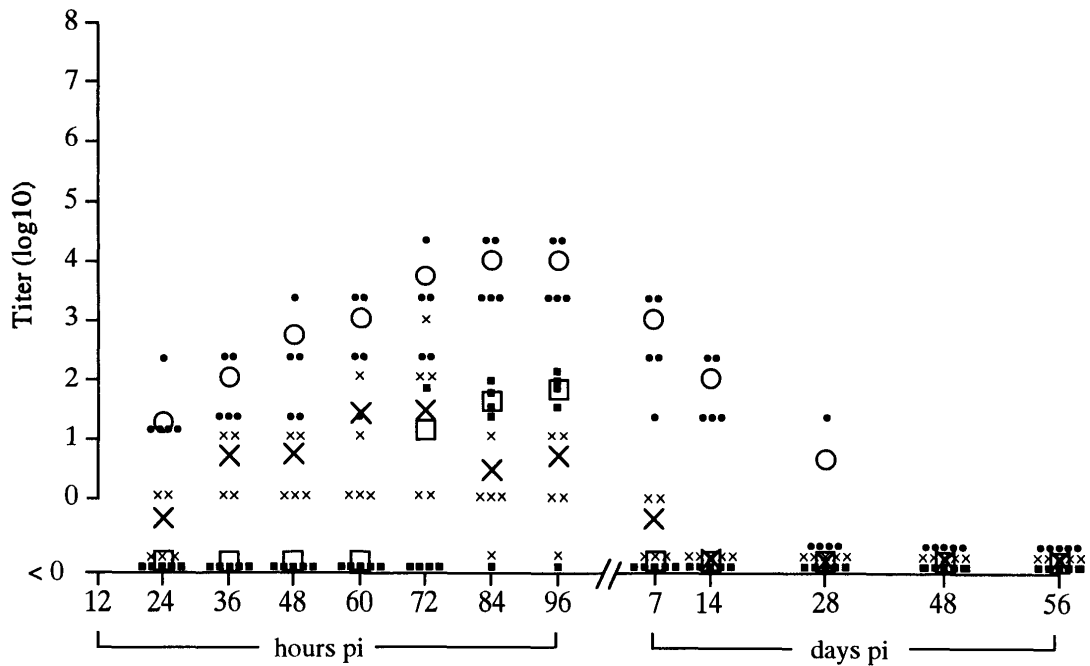


Fig. 3 . Comparison of the sensitivity of the detection of Aichi/68 (H 3 N 2 ) virus infection among the three different assays. For virus isolation and detection of the NS gene or the NS 1 antigen, serial dilutions of nasal washes from mice infected with Aichi/68 (H 3 N 2 ) were tested by three different assays.  $\square$  indicates the average PFU titer of nasal washes by plaque assay,  $\blacksquare$  indicates the PFU titer of each mouse sample.  $\times$  indicates the average titer of nasal washes by RT-PCR,  $\times$  indicates the titer of each mouse sample.  $\circ$  indicates the average titer of nasal washes by immuno-PCR,  $\bullet$  indicates the titer of each mouse sample. All control samples that were collected from mice injected with PBS showed negative (data not shown).

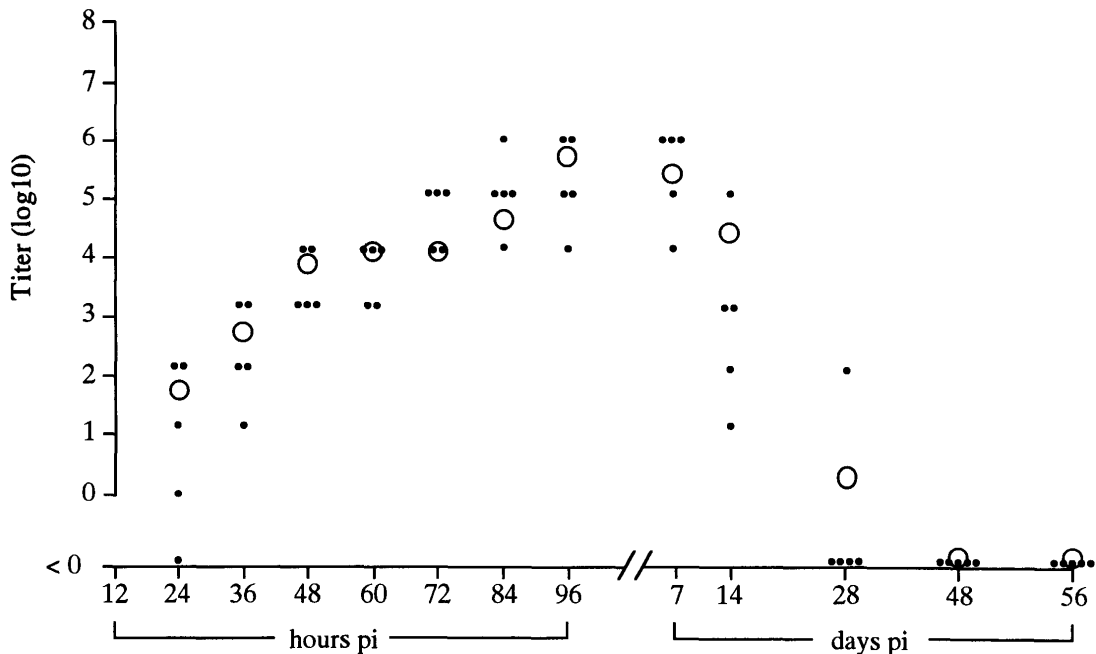


Fig. 4. The HA antigen detection from the nasal washes of mice infected with Aichi/68 (H 3 N 2 ) virus by immuno-PCR. For the detection of the HA antigen, serial dilutions of nasal washes from mice infected with Aichi/68 (H 3 N 2 ) were tested by immuno-PCR.  $\circ$  indicates the average titer of nasal washes,  $\bullet$  indicates the titer of each mouse sample. All control samples from mice infected with PBS showed negative (data not shown).

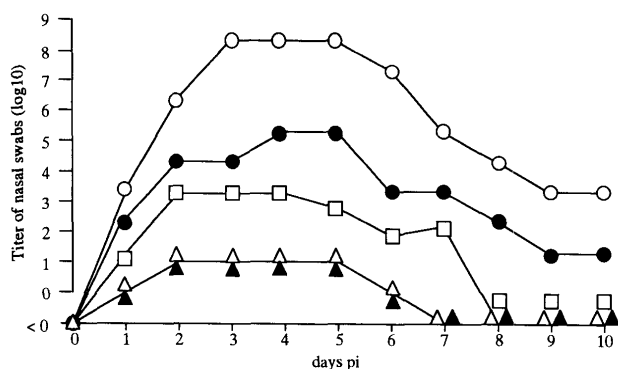


Fig. 5. Detection of equine influenza virus infection in the nasal swabs of a horse infected with Eq/La Plata/93 (H3N8) by plaque assay, RT-PCR, and immuno-PCR. Serial dilutions of nasal swabs from experimentally infected horse were tested and the titers were shown. □ indicates the infectivity titers of nasal swabs by plaque assay. ▲ and △ indicate the titers of detection of the NS and the NP genes by RT-PCR, respectively. ● and ○ indicate the titers of detection of the NS 1 and the HA antigens by immuno-PCR, respectively.

immuno-PCR was  $10^{3.0}$  times higher than that of RT-PCR. Results of the immuno-PCR for the detection of the HA antigen from the nasal washes shown in Fig. 4. The sensitivity of the immuno-PCR for the detection of the HA antigen were  $10^{1.5}$  times higher than that of the NS 1 antigen from the nasal washes of mice at 96 hr pi. The NP gene was also detected from the nasal washes of mice with similar sensitivity to that of the NS gene detection (data not shown). Lung washes from the mice infected with Aichi/68 virus were tested by immuno-PCR, RT-PCR, and plaque assay. Similar detection patterns of positive were observed in the three different methods although the sensitivities were different (data not shown).

To evaluate the practical availability of immuno-PCR, the nasal swabs of an horse experimentally infected with Eq/LP/93 virus were examined. As shown in Fig. 5, infectivity titers in the nasal swabs were up to  $1.9 \times 10^{3.0}$  pfu/0.1 ml and viruses were isolated from 1 to

8 days pi. Immuno-PCR and RT-PCR detected virus antigens and genomes, respectively, in early phase of infection. The titers of the immuno-PCR for the detection of the NS 1 antigen were  $10^{4.0}$  times higher than that of the NS gene detection by RT-PCR on day 4-5 pi. The sensitivity of the immuno-PCR for the detection of the HA antigen was  $10^{3.0}$  times higher than that of the NS 1 antigen and  $10^{7.0}$  times higher than that of the detection of the NS gene or the NP genes by RT-PCR.

### Discussion

Since horses for race or breeding are now transported worldwide by airplanes rapidly and frequently, the risk of transmission of equine influenza virus from the prevalent area is increasing. On the importation of horses, rapid and sensitive method for diagnosis of influenza has been demanded to disclose infected animals at the quarantine. The present results indicate that virus antigen detection by immuno-PCR is promising as a highly sensitive and rapid method for diagnosis of equine influenza.

An immuno-PCR assay based on biotinylated anti-species antibodies and the primers from the ori gene of pUC19 provided a practical means of detecting influenza virus infection with a higher sensitivity than those of plaque-forming assay and RT-PCR. The sensitivity and specificity of the present method are comparable favorably with those reported previously<sup>8,9,11,13,20</sup>.

Detection of influenza virus genes by RT-PCR has been reported as a sensitive method for diagnosis of human influenza<sup>3,10,15,17,19</sup>. In the present study, virus was first recovered at 72 hr pi from one of the five mice while viral antigens and genomes were detected from the same samples of mice at earlier stage of infection by immuno-PCR and RT-PCR, respectively. No more infectious virus was detected



in the nasal or lung washes by the virus isolation assay after 96hr pi, while the NS 1 antigen was detected by immuno-PCR until 2 wks pi and the NS gene was detected by RT-PCR until 1 wk pi. All samples negative by immuno-PCR showed negative by virus isolation as well. It was thus shown that the immuno-PCR was more sensitive than conventional methods and RT-PCR. At the practical use of immuno-PCR in the nasal swabs of an horse infected with Eq/LP/93, both to HA and NS 1 antigens were detected at enough titers by immuno-PCR.

It is, thus, concluded that immuno-PCR is more sensitive than any existing tests and useful for rapid diagnosis of equine influenza. Detection of the NS 1 antigen by immuno-PCR is recommended for diagnosis of equine influenza virus infection using nasal swab samples as a highly sensitive and specific method since the NS 1 is antigenically common among influenza A viruses<sup>2)</sup> and produced only in the infected cells. NS 1 antigen-positive samples by immuno-PCR then could be applied for the HA antigen detection to determine the HA subtype of the causative virus.

#### Acknowledgements

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