

Serosurveillance and establishment of a reverse transcription-polymerase chain reaction assay for bovine parainfluenza virus type 5

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Abstract : Bovine parainfluenza virus type 5 (bPIV5) was isolated from cattle with downer cow syndrome in 2012, and included both respiratory and neurotropic pathogens from a variety of animals. In the current study, we conducted serosurveillance using sera obtained from seven Korean farms and optimized a reverse transcription-polymerase chain reaction (RT-PCR) assay to detect bPIV5. The overall seropositive rate for Korean cattle was 21.4% (163/760). A farm located near the city of Milyang in Gyeongnam province had a markedly elevated seropositive rate for bPIV5 compared to that of the other six farms. The regional seropositive rates were 4.2% (8/192) for Haman, 19.5% (18/55) for Hwasung, 73.9% (65/88) for Milyang, 26.0% (50/192) for Namwon, 1.0% (1/96) for Ulsan, 13.5% (13/96) for Yeongju, and 32.7% (8/41) for Yongin. The sensitivity and specificity of three RT-PCR primer sets used to amplify the conserved fusion gene of bPIV5 were also evaluated. An RT-PCR assay using the bPIVFR3 primer set was 10-fold more sensitive than the assays using the two other primer sets and did not result in non-specific amplification. These results demonstrated that the bPIVFR3 primer set can be used to detect bPIV5.

Keywords : bovine, parainfluenza virus 5, reverse transcription-polymerase chain reaction, serosurveillance

Introduction

The family *Paramyxoviridae* is composed of several genera and gives rise to a number of important human and animal diseases, including measles, human parainfluenza viruses (hPIV), respiratory syncytial virus (RSV), Newcastle disease virus, and parainfluenza virus type 5 (PIV-5/SV-5) [11]. Parainfluenza virus 5 (PIV5), previously known as simian virus 5, belongs to the *Rubulavirus* genus within the subfamily *Paramyxovirinae*, and includes a number of respiratory and neurotropic pathogens found in a variety of animals, as well as humans [12]. PIV5 has a non-segmented, negative-strand RNA genome of approximately 15,000 nucleotides and contains seven genes that encode the following viral proteins: nucleocapsid (N), phosphor (P), matrix, fusion (F), small hydrophobic, hemagglutinin-neuraminidase (HN) and large protein [15, 17]. Human PIV5 (hPIV5) has been isolated from clinical samples proven the neutralizing antibody against PIV5 [8, 9]. Canine PIV5 (cPIV5) is considered to be one of the important viral agents causing canine respiratory disease, including bronchitis and bronchiolitis, in combination with certain bacterial agents [2, 3]. In addition, porcine PIV5 has been isolated from the lung of fetuses within sows infected with porcine reproductive and respiratory syndrome virus

(PRRSV), and is considered to be a mild pathogen in pigs [13]. Bovine PIV5 (bPIV5) infection was reported in Korean cattle showing neurological symptoms and the biological/molecular characterization of bPIV5 isolates was reported in 2014 [20].

Several methods of detecting antibodies to PIV are available, including enzyme-linked immunosorbent assay (ELISA), hemagglutination inhibition (HI), complement fixation, and hemadsorption inhibition assays (HADI) [10]. ELISA is by far the most sensitive and can be applied to a large number of serum samples, but it is the least specific assay and detects antibody titers against other type of hPIV. In addition, no species-specific ELISA to detect antibodies against PIV5 has been developed. The HADI assay, which is based on the biological properties of PIV5, can be applied to detect antibodies, but requires a cell culture system, erythrocytes, and a skilled technician [19]. The HI test is considered the standard method for detecting PIV5 antibodies [3]. Therefore, in this study, the HI test was used to screen for bPIV5 antibodies and confirm bPIV5 infection in Korean cattle.

We performed a seroepidemiological survey of bPIV5 and also established a diagnostic tool, an optimized reverse transcription-polymerase chain reaction (RT-PCR) assay, to facilitate detection of bPIV5 infection in cattle. These contributions

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are important because data on bPIV5 incidence can be used for vaccine development and initiating measures to block virus transmission. Therefore, in this study, we conducted a serological survey to determine the prevalence of antibodies against bPIV5 in cattle and optimize the reaction conditions for a bPIV5 RT-PCR assay.

Materials and Methods

Collection of sera and preparation of bPIV5

In total, 760 blood samples were collected from Korean cattle on seven farms from four provinces in South Korea. Clotted blood samples were separated by centrifugation, and the sera were stored at -20°C until use. Bovine PIV5 (strain QIA-B1201) isolated from a farm field in 2012 was propagated in Vero cells. Vero cells were regularly maintained in α -minimum essential medium (α -MEM) supplemented with 5% fetal bovine serum, penicillin (100 IU/mL), streptomycin (10 $\mu\text{g}/\text{mL}$), and amphotericin B (0.25 $\mu\text{g}/\text{mL}$). To propagate bPIV5, Vero cells grown in α -MEM were washed three times with phosphate-buffered saline (PBS), and then inoculated. After adsorption, α -MEM was added, and the cells were incubated until a 90% cytopathic effect was observed in the infected Vero cells. After harvesting, the antigen was used in the HI test and for detecting antigens via RT-PCR. The Akabane virus, Aino virus, Chuzan virus, bovine ephemeral fever virus (BEFV), bovine viral diarrhea virus (BVDV), and infectious bovine rhinotracheitis virus (IBRV) were used as reference strains to test the specificity test of the RT-PCR assay.

Hemagglutination assay (HA) and HI test

The HA test was performed by preparing a serial twofold dilution of bPIV5 (strain QIA-B1201) into 50 μL of PBS (pH 7.2) with 50 μL of 0.6% chicken erythrocytes. The HA titer was expressed as the reciprocal of the highest dilution of bPIV5 showing complete hemagglutination. The HI test was performed in 96-well microplates according to a modified method. Briefly, to remove non-specific inhibitors, 20 μL of serum were mixed with 180 μL of 25% kaolin and incubated for 30 min. After pipetting, the kaolin was removed by centrifugation at $2,500 \times g$ for 15 min in a microfuge. The result-

ant clear supernatant was mixed with 5 μL of packed chicken erythrocytes to remove any natural agglutinins. After incubation for 1 h at 37°C , the treated serum was separated from the erythrocytes by centrifugation. For the HI test, 4–8 HA units of bPIV5 (in 25 μL) were added to 25 μL of the treated serum. After incubation for 1 h at room temperature, 50 μL of 0.6% chicken erythrocytes were added, and the microplates were incubated at 37°C for 1 h. The HI titer was expressed as the reciprocal of the highest dilution of serum showing complete inhibition of hemagglutination. Serum samples showing an HI titer $\geq 1 : 20$ were considered positive.

Selection of primers and RT-PCR

Three different primer pairs were designed based on the highly conserved F gene from strain QIA-B1201 (GenBank accession no. KR061998). Viral RNA was extracted from QIA-B1201 using an RNA extraction kit (Bioneer, Korea) according to the manufacturer's instructions. The extracted RNA was eluted in 50 μL of RNase- and DNase-free water. The RT-PCR assay was performed with specific primers (PIV5F1, PIV5R1, PIV5F2, PIV5R2, PIV5F3, and PIV5R3) that amplify the F gene of bPIV5 (Table 1). RT-PCR was carried out in a reaction mixture containing 10 μL of denatured RNA, 1 μL of each primer (50 pmol), 10 μL of $5\times$ buffer (12.5 mM MgCl_2), 2 μL of dNTP mix, 2 μL of enzyme mix (reverse transcriptase and *Taq* polymerase), and 24 μL of distilled water (Qiagen, Hilden, Germany). The cycling profile consisted of cDNA synthesis at 42°C for 30 min, followed by 35 cycles at 95°C for 30 sec, 52°C for 30 sec, and 72°C for 45 sec, with a final extension at 72°C for 5 min. The PCR products were visualized using gel electrophoresis on 1.8% agarose gels containing ethidium bromide.

Results

Serosurveillance of bovine PIV5

The results of bPIV5 serosurveillance in Korean cattle were shown in Figures 1 and 2. An HI titer of $\geq 1 : 20$ was considered to be positive for bPIV5. The overall bPIV5 seropositive rate in Korean cattle was 21.4% (163/760). One farm located near the city of Milyang in Gyeongnam province showed a markedly elevated seropositive rate compared

Table 1. List of the oligonucleotide primers used for RT-PCR assays against the F gene of bPIV5

Primer designated	Oligonucleotide sequence (5'-3')	nucleotide position	Size of amplicon
bPIV5F1	GGCAGGAGCAGGCAGCCTTG	42–61*	213
bPIV5R1	CTCACCGATCGGCTGTAGGAG	255–235	
bPIV5F2	CGGAGGAGACGCCGGTTTGC	291–311	402
bPIV5R2	GGTACTCCCCAGTAGGATCC	693–674	
bPIV5F3	CCTTCTCTCCAGTGGTGGG	1069–1089	299
bPIV5R3	CACCGCAGCTAGATTCTGGG	1368–1359	

*GenBank accession no. KR061998. RT-PCR, reverse transcription-polymerase chain reaction; F, fusion protein; bPIV5, bovine parainfluenza virus type 5.

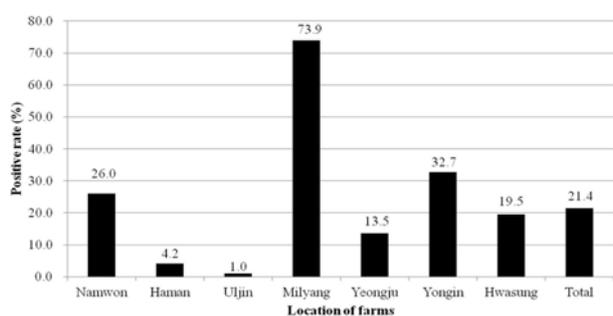


Fig. 1. Distribution of seropositivity for bovine PIV5 according to the farm.

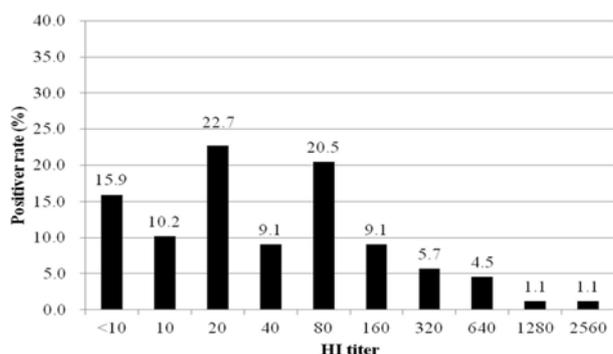


Fig. 2. Frequency distribution of HI titers against bovine PIV5 on Milyang farm. Titers < 1 : 20 were considered negative.

to the remaining six farms. Seropositive rates according to region were 4.2% (8/192) in Haman, 19.5% (18/55) in Hwasung, 73.9% (65/88) in Milyang, 26.0% (50/192) in Namwon, 1.0% (1/96) in Uljin, 13.5% (13/96) in Yeongju, and 32.7% (8/41) in Yongin (Fig. 1). At the highly seropositive farm in Milyang, the most frequent HI titer was 1 : 20 (22.7%) (Fig. 2).

Sensitivity and specificity

An RT-PCR assay for the detection of bPIV5 was carried out using three primer sets to optimize assay sensitivity. The bPIV5 culture supernatant (QIA-B1201; $10^{7.0}$ TCID₅₀/mL) was subjected to a sensitivity test. The eluted RNA was serially diluted 10-fold to 10^{-5} and then used for one-step RT-PCR. Sensitivity of the RT-PCR assay was expressed as infectivity titer equivalent. As shown in Figure 3, the detection limit of the RT-PCR assays using primer sets bPIVFR1 and 2 was 2×10^3 TCID₅₀/reaction in each assay, whereas the sensitivity of the assay using primer set bPIVFR3 was 2×10^2 TCID₅₀/reaction. Overall, the bPIVFR3 primer set was 10-fold more sensitive than bPIVFR1 or bPIVFR2. As shown in Figure 4, RT-PCR using the bPIVFR1 primer set resulted in non-specific reactions in Aino virus, BEFV, and Chuzan virus, and amplified a band of roughly the same size as bPIV5 in IBRV. However, RT-PCR with primer sets bPIVFR2 and 3 did not result in any non-specific reaction. Although RT-PCR with any of the three primer sets was able

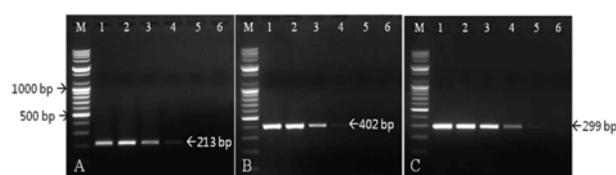


Fig. 3. Sensitivity test using three primer sets against bovine PIV5. (A) bPIVFR1. (B) bPIVFR2. (C) bPIVFR3. M, 1-kb DNA ladder; Lane 1–6, 10-fold dilution of bPIV5.

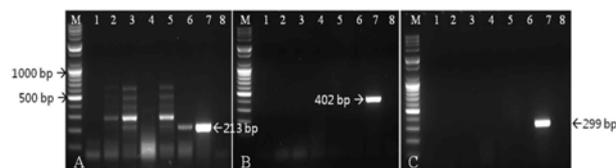


Fig. 4. Specificity test using three primer sets against bovine PIV5. (A) bPIVFR1. (B) bPIVFR2. (C) bPIVFR3. M, 1kb DNA ladder; Lane 1, Akabane virus; Lane 2, Aino virus; Lane 3, bovine ephemeral fever virus; Lane 4, bovine viral diarrhea virus; Lane 5, Chuzan virus; Lane 6, infectious bovine rhinotracheitis virus; Lane 7, bovine PIV5; Lane 8, negative.

to amplify bPIV5 with high sensitivity, only bPIVFR3 was acceptable in terms of both sensitivity and specificity.

Discussion

Since the first hPIV was reported in the late 1950s, four major hPIV serogroups have been identified based on antigenic and genetic characteristics. Serogroups hPIV1 and 3 gave rise to *Respirovirus*, and hPIV2 and 4 have been clustered into *Rubulavirus*. These hPIVs have caused respiratory illness in children less than five years of age [21]. Viral antigens commonly associated with respiratory disease in cattle include IBRV, bovine respiratory syncytial virus (BRSV), and parainfluenza virus type 3 (PIV3) [16]. It is notable that PIV3 alone does not usually produce severe disease in cattle. However, combination with certain bacterial agents, such as *Mannheimia* and *Mycoplasma*, promotes the formation of the enzootic pneumonia complex. PIV3 infection is thought to be stress-related, perhaps due to mixing of animals of different age groups, transport stress, or winter housing under poor hygienic conditions. However, PIV5, known as cPIV2 in a veterinary context, is less well understood in cattle throughout the world.

The seroprevalence of cPIV2 in dogs has been estimated to range from 10.4% in Canada to 28.9% in Czechoslovakia [7, 22]. It is reported that about 29% of human serum samples had VN antibodies against hPIV5, suggesting that some humans have been exposed to hPIV5 from dogs [6]. However, no serosurvey data are available regarding bPIV5 in cattle populations in any part of the world. In the present study, 12.4% (163/760) of Korean cattle from seven farms were seropositive for bPIV5. The cattle population (n = 672)

in six of seven farms showed few HI antibodies to bPIV5. Interestingly, one farm located in Milyang city showed markedly elevated seroprevalence (73.9%) and 42% of cattle had an antibody titer $> 1 : 80$ (Fig. 2), indicating that a high proportion of the cattle had been recently exposed to bPIV5 and that the virus had circulated within the cattle population. In addition, most of the cattle on the farm had experienced a respiratory illness and some cattle were found to be persistently infected with BVDV. We assume that close contact amongst the farmer, dogs, and cattle may be a contributing factor to the exposure of cattle to bPIV5 from dogs or humans. Most of the dogs reared on cattle farms are inoculated with a multivalent vaccine containing cPIV2. A previous study reported that PIV5 was detected in dogs at post-inoculation day 3 using an RT-PCR assay [6]. Many diseases in humans are associated with hPIV5 and antibodies against hPIV5 are detected in human populations [14].

Virus isolation by cell culture and direct immunofluorescent antibody assay are the most commonly used technique for detecting PIV5. However, the above-mentioned methods have significant limitations in terms of sensitivity [18]. Therefore, RT-PCR was explored as a means to overcome limitations on sensitivity and specificity and provide rapid results. Conserved sequences within the N, P, F, and HN regions have been used for amplifying PIVs [4, 13]. However, the use of a poor primer set can result in low sensitivity or false-positive reactions in the RT-PCR assay. In the present study, after considering the length of the amplified fragment, three primer sets based on the F gene of QIA-B1201 were used to evaluate sensitivity and specificity using 10-fold serial dilutions of viral RNA extracted from QIA-B1201. The results showed that the bPIVFR3 primer set was 10-fold more sensitive (2×10^2 TCID₅₀/reaction) than the bPIVFR1 or bPIVFR2 primer set, and was able to amplify only the F gene of bPIV5 without any non-specific reaction. The detection limit was consistent with previous reports [1, 4, 6]. Cell lines used in laboratories can be persistently infected with PIV5 and these cells typically do not show detectable cytopathic effects [5]. Therefore, the RT-PCR assay using the bPIVFR3 primer set may be useful in the diagnosis of PIV5 infection in animals and when checking cell lines in laboratories.

In conclusion, based on the serosurveillance of bPIV5, a small number of Korean cattle have been exposed to bPIV5, which may cause respiratory disease under poor hygienic conditions. The RT-PCR assay described here was both sensitive and specific for the detection of bPIV5. However, we did not attempt to apply this RT-PCR assay to a large number of field samples. Therefore, further studies are required to determine whether this RT-PCR assay provides robust results from several types of specimen.

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