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ORIGINAL ARTICLE

Full genomic amplification and subtyping of influenza A virus using a single set of universal primers

Emi Inoue[1], Xiaofeng Wang[1,3], Yoshiaki Osawa[1] and Katsunori Okazaki[1,2]

[1]Laboratory of Microbiology and Immunology, Faculty of Pharmaceutical Sciences, and [2]Division of Medicinal Science, The Research Institute of Personalized Health Sciences, Health Sciences University of Hokkaido, Ishikari-Tobetsu, Hokkaido 061-0293, Japan, and [3]Department of Microbiology, Medical College of Qingdao University, Quingdao 266021, China

ABSTRACT

Influenza A virus has eight-segmented RNA molecules as a genome and, among all strains of the virus, both ends of each segment have 13 and 12 nucleotide sequences conserved. In the present study, a simple RT-PCR method to amplify all eight segments of the virus and determine the HA and NA subtype using a single primer set based on the conserved terminal sequences has been established. This method is also capable of detecting subgenomic defective interfering RNA of the influenza A virus. Since the primers used here cope with each and every RNA segment of influenza A virus, this simple RT-PCR method is valuable not only for cloning each gene of the virus, but also for identifying subtypes, including subtypes other than 16 HA and 9 NA subtypes.

Key words genomic amplification, influenza A virus, single primer set, subtyping.

Influenza A viruses commonly infect humans, pigs, horses and a variety of avian species. The virus has eight-segmented negative-sense RNA molecules as a genome. Two of the eight segments encode surface glycoproteins, HA and NA, which are classified into 16 and 9 subtypes, respectively. Every subtype of glycoprotein has been detected in the pool of avian influenza viruses (1), although only limited combinations of subtypes are currently circulating in mammals.

In April 2009 a novel influenza A virus (H1N1) containing a combination of swine, avian and human virus gene segments emerged in humans (2, 3) and spread worldwide, resulting in the declaration of the pandemic (H1N1) 2009 by the World Health Organization in July. It was thought that avian influenza viruses could be transmitted to humans only through coinfection and genetic reassortment of avian and swine or human viruses in pigs, and this was thought to be true of the pandemic (H1N1) 2009 strain, which shows moderate virulence (4–8). It is, however, also known that avian influenza viruses can be transmitted directly to humans, for example, the H5N1 and H9N2 viruses emerged in humans in Hong Kong in 1997 (9–13) and the H7N7 virus in the Netherlands in 2003 (14). The highly pathogenic H5N1 virus re-emerged in Hong Kong in 2003 (15), and since then more than 400 people have been infected, more than 250 having died of the infection (16). Thus, it is important that rapid, sensitive and specific assays are designed for use when outbreaks of the disease occur.

The HA and NA subtypes of influenza A viruses are identified by the HI and NI tests using a panel of antisera against each HA and NA subtype (17). Genetic analysis with RT-PCR has been attempted to discriminate the subtypes or a reassortment event by using subtype or segment specific multiple primer sets (1, 18–21). The RNA segments of influenza A viruses have 13 and 12 conserved

Correspondence
Katsunori Okazaki, Department of Microbiology and Immunology, Faculty of Pharmaceutical Sciences, Health Sciences University of Hokkaido, Ishikari-Tobetsu, Hokkaido 061-0293, Japan.
Tel/fax: +81 133 23 1333; email: kokazaki@hoku-iryo-u.ac.jp

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List of Abbreviations: AMV, avian myeloblastosis virus; DI, defective interfering; HA, hemagglutinin; HI, hemagglutination inhibition; M, matrix; NA, neuraminidase; NI, neuraminidase inhibition; NP, nucleoprotein; NS, nonstructural; RT, reverse transcriptase.

sequences at both the 5′- and 3′-ends (Uni13 and Uni12), respectively (22, 23). Although many research groups have used both of the conserved sequences to design primers for RT-PCR, in practice the sequences alone are too short to be used as universal primers for amplifying each segment. Recently, modified oligonucleotides have been reported to amplify all segments of influenza A viruses. However, different sets of specific primers are required to amplify each segment and an additional four sets are needed for amplifying different subtypes of the NA genes (24).

The present study aimed at establishing a much simpler RT-PCR method that enables all eight segments of the influenza A virus genome to be amplified and the HA and NA subtypes to be determined by a single primer set.

MATERIALS AND METHODS

Viruses

The virus strains used in this study are A/Aichi/2/68 (H3N2), A/swan/Shimane/499/83 (H5N3), A/duck/Czeckoslovakia/56 (H4N6), A/chicken/Germany/N/49 (H10N7) and a reassortant virus A/NWS/33 x A/tern/Australia/G70c/75 (H1N9). Viruses were propagated by inoculation into the allantoic cavities of 10-day-old embryonated chicken eggs.

RNA extraction and RT-PCR

vRNA was extracted from the allantoic fluid by use of the QuickGene RNA tissue kit S II (Fujifilm, Tokyo, Japan) according to the manufacturer's instructions. The RNA (5 μl) was transcribed into cDNA with AMV RT (Promega, Madison, WI, USA) and the primer FWuni12: 5′-CTGATCTAGACC-TGCAGGCTCAGCAAAAGCAGG-3′, which contains *Xba* I, *Pst* I and *Sse8387* I recognition sequences in upstream of complementary sequence to Uni12 (underlined). The reaction mixtures, containing 5 μl of 10 μM FWuni12 and 5 μl of distilled water, were heated at 70°C for 10 min and chilled on ice for 5 min. Then, 5 μl of first strand 5x buffer, 40 U of RNasin (Promega), 2.5 μl of 40 mM sodium pyrophosphate and 30 U of AMV RT were added to the mixtures. The reaction was performed at 42°C for 60 min. Second-strand cDNA was synthesized from the first-strand cDNA by using DNA polymerase I (Promega) and the primer RVuni13: 5′-CGTGGTACCATGGTCTAGAGT-AGTAGAAACAAGG-3′, which contains *Kpn* I, *Nco* I and *Xba* I recognition sequences and a complementary sequence to Uni13 (underlined). The second reaction mixtures, containing 25 μl of the first-strand cDNA, 40 μl of second strand 2.5x buffer, 5 μl of acetylated BSA, 3 μl of 10 μM RVuni13, 0.8 U of RNaseH (Promega) and 26.5 μl of distilled water, were incubated at 37°C for 1 hr to remove vRNA. Then, the mixtures were incubated with 23 U of DNA polymerase I at 14°C for 3 hr according to the manufacturer's instructions, with a slight modification to generate double-strand cDNA which carries an elongated priming site suitable for PCR. The double-strand cDNA was amplified by using the AccuPrime *Pfx* DNA polymerase (Invitrogen, Carlsbad, CA, USA) and a set of primers, FWuni12 and RVuni13. The PCR mixtures contained 5 μl of 10x buffer, 1.5 μl of 10 μM each primer, 30 μl of the double-strand cDNA, 0.5 μl of AccuPrime *Pfx* DNA polymerase and 11.5 μl of distilled water. The first cycle of the amplification program consisted of a 1-min period at 94°C and was followed by 30 cycles with the following conditions: 94°C for 30 sec, 60°C for 30 sec and 72°C for 3 min. The program ended with one cycle at 72°C for 5 min.

Direct sequencing of the amplification products

The amplification products were separated by electrophoresis using 1.5% agarose gels and purified using the Wizard SV Gel and PCR Clean-Up System (Promega). The amplicons were directly sequenced with BigDye Terminator ver1.1 Cycle Sequencing Kit (Applied Biosystems, Foster, CA, USA) using the primers FWuni12 and RVuni13. The cycle sequencing mixtures contained 10 ng of the PCR product, 2 μl of 5x buffer, 3.2 pmol of the primer, 4 μl of Ready Reaction Mix and distilled water in a total volume of 20 μl. The first cycle of the program consisted of a 1-min period at 95°C and was followed by 25 cycles with the following conditions: 95°C for 30 sec and 60°C for 4 min. The sequences were analyzed with an ABI Prism 310 Genetic Analyzer (Applied Biosystems).

Cloning and plasmid sequencing

The amplification products corresponding to the polymerase genes were phosphorylated by using T4 polynucleotide kinase (Takara Bio, Othu, Japan) according to the manufacturer's instructions and cloned into *Eco*R V site of pBluescript II KS(+) plasmid (Stratagene, La Jolla, CA, USA). The nucleotide sequences of the inserts were analyzed with BigDye Terminator ver1.1 Cycle Sequencing Kit using FWuni12/RVuni13 or T7/T3 promoter primers. The first cycle of the program consisted of a 1-min period at 95°C and was followed by 25 cycles with the following conditions: 95°C for 30 sec, 48°C for 30 sec and 72°C for 4 min.

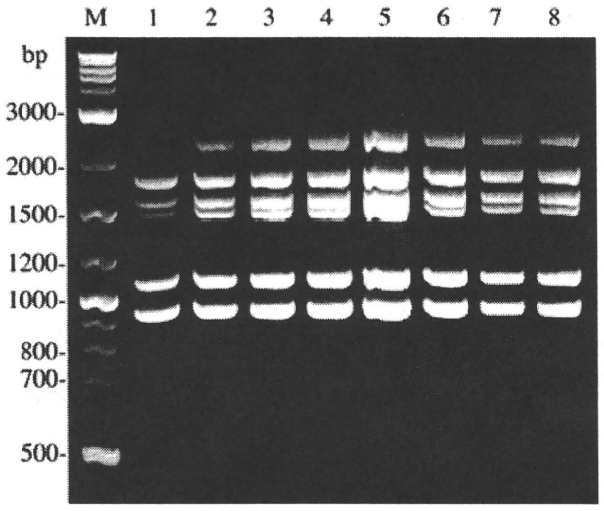

Fig. 1. Full length amplification of all eight segments of A/Aichi/2/68 (H3N2) by RT-PCR. The annealing temperature was varied from 45°C to 70°C. Lane M, 2-Log DNA ladder (New England Biolabs, Ipswich, MA, USA), lanes 1 to 8, annealing at 70°C, 68.3°C, 65.3°C, 60.8°C, 54.5°C, 49.9°C, 46.5°C and 45°C.

RESULTS

Amplification of A/Aichi/2/68 (H3N2) genome

The PCR conditions for annealing temperature was varied from 45°C to 70°C to amplify the genome of A/Aichi/2/68 (H3N2). As shown in Fig. 1, six fragments of 2.3 kb, 1.8 kb, 1.6 kb, 1.5 kb, 1.1 kb and 0.9 kb in length were successfully amplified with all annealing temperatures, and the most effective amplification was observed with the annealing step at 60.8°C. These fragments appeared to correspond to the polymerases (PB1, PB2 and PA), HA, NP, NA, M and NS genes, respectively, in length. The amplification products, except for 2.3 kb-one, were excised from the gel and directly sequenced by use of FWuni12 and RVuni13 as primers. The nucleotide sequences found in the products coincided with those of both ends of the HA, NP, NA, M and NS genes of A/Aichi/2/68 (H3N2), respectively.

Cloning of the polymerase genes of A/Aichi/2/68 (H3N2)

To detect the PB1, PB2 and PA genes of the virus, the 2.3 kb-fragment was cloned into the *Eco*R V site of pBluescript II KS(+) plasmid and sequenced. Out of 62 clones tested, 2 (3.2%), 6 (9.7%) and 54 (87.1%) clones were found to possess both ends of the PB1, PB2 and PA genes, respectively (Table 1). These results indicate that the full length of all eight segments of A/Aichi/2/68 (H3N2) can be amplified and sequenced with this single primer set.

Table 1. Frequency of the polymerase genes of A/Aichi/2/68 (H3N2) cloned into the plasmid

	Number of clones (%)		
	PB1	PB2	PA
Exp 1	1 (3.3)	2 (6.7)	27 (90.0)
Exp 2	1 (3.1)	4 (12.5)	27 (84.4)
Total	2 (3.2)	6 (9.7)	54 (87.1)

Amplification of the genes encoding different subtypes of NA

Hoffman *et al.* designed seven sets of universal primers to amplify all but the NA gene (24). Although the N1, N2, N4, N5 and N8 subtype NA genes were able to be amplified with a single set of primers, other subtype NA genes were required to subtype specific primer sets. Since the N2 NA gene had been successfully amplified and sequenced, we attempted to amplify the different NA genes of N3, N6, N7 and N9 subtypes with a single primer set. The PCR condition was performed with annealing step at 60°C for 30 sec. Figure 2 shows amplification of the whole genome of different influenza A virus strains including each of the

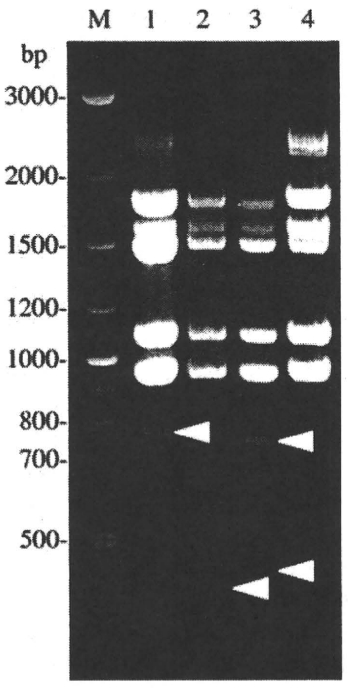

Fig. 2. Amplification of the whole genome of influenza A viruses representing four different NA subtypes. Lane M, 2-Log DNA ladder, lanes 1 to 4, A/swan/Shimane/499/83 (H5N3), A/duck/Czeckoslovakia/56 (H4N6), A/chicken/Germany/N/49 (H10N7) and a reassortant virus A/NWS/33 x A/tern/Australia/G70c/75 (H1N9). Arrowheads show the extra bands, which are smaller than the NS gene.

NA gene using the primer set. Although the polymerase genes of A/swan/Shimane/499/83 (H5N3), A/duck/Czeckoslovakia/56 (H4N6) and A/chicken/Germany/N/49 (H10N7) were detected only faintly, the HA, NP, NA, M and NS genes were sufficiently amplified. Although no increased amplification of the polymerase genes was observed with the longer extension time, re-amplification of the 2.3 kb faint bands gave distinct bands at the position of the polymerase genes (data not shown). The amplification products of A/swan/Shimane/499/83 (H5N3), A/duck/Czeckoslovakia/56 (H4N6) and A/chicken/Germany/N/49 (H10N7) contained prominent extra bands which migrated faster than those of the NS gene segments. The amplification products corresponding to the NA genes were excised from the gel and sequenced with the primers. The nucleotide sequences of the amplicons coincided with those of each subtype of the NA gene. We also confirmed the appropriate nucleotide sequences of the amplicons corresponding to the H1, H4, H5 and H10 subtype HA genes. These findings indicate that the single set of FWuni12/RVuni13 primers accomplished amplification of the whole genome of all influenza A viruses. This single primer set also enabled the HA and NA subtypes to be defined for all influenza A viruses.

Detection of DI RNA segment of A/duck/Czeckoslovakia/56 (H4N6)

To ascertain the nature of the extra band which migrated faster than the NS gene, the 0.4 kb-band of A/duck/Czeckoslovakia/56 (H4N6) was directly sequenced by use of FWuni12 and RVuni13 as primers. The nucleotide sequences found in the product coincided with both ends of the PB1 gene. It is, therefore, presumed that the extra band comes from the PB1 gene and lacks the mid-position of the gene. In order to confirm this presumption, the 2.3 kb-fragment of the virus was cloned and examined for which gene was contained in the plasmid. Out of 46 clones sequenced, none of the PB1 gene was found, while 22 (47.8%) and 24 (52.2%) clones possessed both ends of the PB2 and PA genes, respectively (Table 2). This result probably indicates that the virus stock of A/duck/Czeckoslovakia/56 (H4N6) contains many DI particles, which carry internal deletion in the PB1 gene segment. Other extra bands of A/swan/Shimane/499/83 (H5N3) and A/chicken/Germany/N/49 (H10N7) did not give significant sequences by use of FWuni12/RVuni13 primers.

Table 2. Frequency of the polymerase genes of A/duck/Czeckoslovakia/56 (H4N6) cloned into the plasmid

	Number of clones (%)		
	PB1	PB2	PA
Exp 1	0 (0.0)	11 (57.9)	8 (42.1)
Exp 2	0 (0.0)	11 (40.7)	16 (59.3)
Total	0 (0.0)	22 (47.8)	24 (52.2)

DISCUSSION

In the present study, we achieved full length amplification of all eight segments of the influenza virus A/Aichi/2/68 (H3N2) genome by using a single primer set. Although a multiplex RT-PCR method, in which 12-mer and 13-mer oligonucleotides complementary to the conserved regions was used to simultaneously amplify all eight RNA segments of equine H7N7 virus, has been described previously (25), there has been no report of this method thereafter. In one recent study all eight segments of different influenza A virus strains were amplified using the segment specific primer sets, but the primers for the NA gene depend on the subtype (24). Our single set of primers was, on the other hand, successful in amplifying every segment of all influenza A viruses examined.

So far, the subtyping of influenza A virus has been carried out with the HI and NI tests, for which a large panel of antisera is needed. Hoffman *et al.* described universal primers for identifying the HA subtype (24). However, at least five sets of primers were required to define the different NA subtypes. It is, therefore, questionable whether such subtype specific primers are applicable for defining a novel subtype of the influenza A virus. Alvarez *et al.* have described one-step RT-PCR, detecting all nine NA subtypes with a single primer set (26). These RT-PCR amplicons, however, include only a part of the NA gene related to oseltamivir resistance. The present procedure is a much more universal and effective tool for amplifying the influenza A virus gene and indentifying all HA and NA subtypes, including unknown ones. We succeeded in amplifying the genome of the novel influenza A (H1N1) virus and in detecting the nucleotide sequence of the HA and NA genes of so-called swine (H1N1) virus.

Segment specific primers may be preferable to universal primers for detecting reassortment events, especially those concerned with the polymerase genes. However, digestion of the 2.3 kb-fragment by restriction enzymes, which recognize the sequences in the universal primers, certainly helps to clone a part of the fragment into the plasmid. Then nucleotide sequencing with T7/T3 promoter primers or restriction fragment length polymorphism analysis can be done to identify the gene segment.

An extra band smaller than the NS gene and possessing both ends of the PB1 gene was found in the amplification products of A/duck/Czeckoslovakia/56 (H4N6) and none of the full length of the gene was cloned in this study.

These results seem to make sense, since influenza virus DI particles possess internal deleted RNA segments which have arisen from the polymerase genes (27–29). Colony hybridization with the central region of the gene as a probe might help to clone the full-length polymerase gene. Since it has been suggested that influenza A virus bearing the subgenomic RNA transmits in nature (27), the detection of the DI RNA may be of use in surveillance studies. We also detected only a small number of the PB1 and PB2 genes of A/Aichi/2/68 (H3N2). Although no extra fragment of A/Aichi/2/68 (H3N2) was found, the inefficient cloning of the genes reflects possible DI RNA.

In conclusion, we have established a novel RT-PCR procedure that enables all eight segments of influenza A virus genome to be amplified by a single primer set. In the context of increasing viral diversity, this universal method is valuable for identifying the HA and NA subtypes, even for novel influenza A viruses. This method was also successful in detecting the subgenomic DI RNA. The simple and universal RT-PCR procedure described here is expected to provide new insights into the epidemiology of influenza A virus as well as into molecular biology, facilitating creation of novel diagnostic agents and vaccines for influenza A virus infection.

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Molecular epidemiology of rotaviruses among healthy calves in Japan: Isolation of a novel bovine rotavirus bearing new P and G genotypes

Masako Abe[a], Naoto Ito[a,b], Shigeki Morikawa[b], Masaki Takasu[c], Tetsuma Murase[c], Takanori Kawashima[d], Yoshihiro Kawai[d], Junko Kohara[e], Makoto Sugiyama[a,b,*]

[a] The United Graduate School of Veterinary Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
[b] Laboratory of Zoonotic Diseases, Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
[c] Theriogenology, Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
[d] Gujo City Veterinary Clinic Center, Gujo, Gifu 501-4297, Japan
[e] Hokkaido Animal Research Center, Shintoku, Hokkaido 081-0038, Japan

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ABSTRACT

A total of 171 fecal specimens collected from healthy calves on a beef farm in Gifu Prefecture, Japan in 2006–2007 were examined for group A rotaviruses by RT-semi-nested PCR targeting the coding region for VP8*. Nine specimens were positive for rotavirus. G and P genotyping indicated that one strain was G10P[11]-like and six strains were considered to be the same unknown G and P genotypes. Among these six untypeable strains, one strain, AzuK-1, was adapted to cell culture and analyzed. Sequence and phylogenetic analyses of the full lengths of VP4 and VP7 genes revealed that AzuK-1 strain is a novel bovine rotavirus bearing new G21 and P[29] genotypes as confirmed by the RCWG. Furthermore, we detected G21P[29] rotaviruses in fecal specimens collected from healthy calves in Hokkaido, Japan during the period from 1997 to 1998. These findings suggest that novel G21P[29] rotaviruses have been widely prevalent among cattle for over 10 years in Japan.

1. Introduction

Group A rotaviruses are known to be major agents of severe acute gastroenteritis in infants and young animals worldwide. Approximately 611,000 children worldwide die from rotavirus diarrhea every year, the numbers of deaths being particularly high in developing countries (Estes, 2001; Glass et al., 2006; Parashar et al., 2006). Even in developed countries, rotavirus remains an important cause of morbidity. For example, in the United States, it has been estimated that rotavirus accounted for 47,000–60,000 hospitalizations each year of children less than 5 years of age, suggesting that 1 in 67 to 1 in 85 children will be hospitalized with rotavirus by the age of 5 years (Malek et al., 2006). Rotavirus-induced diarrhea is therefore a serious public health problem throughout the world.

Rotaviruses are members of the *Reoviridae* family. The virus particles have a triple-layered protein capsid that contains a genome of 11 segments of double-stranded RNA, which encode six viral structural proteins (VP1–4, VP6 and VP7) and six nonstructural proteins (NSP1-NSP6). Each RNA segment is monocistronic, with the exception of segment 11, which has an additional overlapping open reading frame, encoding NSP6. Because of the segmented nature of the genome, infection of one cell with two different rotaviruses could result in a reassortment event, which is known to generate new viruses, so-called "reassortants" with combinations of genome segments from two parental viruses (Palombo, 2001; Ramig, 1997). Reassortment is one of the important mechanisms for generating genetic diversity of rotaviruses and eventually for viral evolution.

VP4 and VP7, which are encoded by segments 4 and 9, respectively, are components of the viral outer capsid. VP4 forms viral spikes that project from the surface of viral particle. VP4 has been shown to participate in several important functions, such as cell attachment, entry into cells, haemagglutination, neutralization, virulence, and protease-enhanced infectivity of rotavirus (Dunn et al., 1995; Estes, 2001; Fuentes-Panana et al., 1995; Kaljot et al., 1988; Ludert et al., 1996; Offit et al., 1986). By protease treatment, VP4 is cleaved into two polypeptides, VP8* and VP5*. VP7, the most abundant external glycoprotein, forms a smooth surface of the virion. VP7 is the major neutralization antigen and is involved in the cell entry process (Fukuhara et al., 1988; Sabara et al., 1985).

* Corresponding author at: Laboratory of Zoonotic Diseases, Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido, Gifu, Gifu 501-1193, Japan. Tel.: +81 58 293 2948; fax: +81 58 293 2948.
E-mail address: sugiyama@gifu-u.ac.jp (M. Sugiyama).

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According to diversities of VP4 and VP7 genes, group A rotaviruses are classified into P and G genotypes. Rotavirus strains with VP4 and VP7 sharing 89% or greater amino acid identities belong to the same P and G genotypes, respectively (Estes, 2001; Gorziglia et al., 1990; Nishikawa et al., 1989). Recently, a novel classification system for rotaviruses has been proposed based on the nucleotide sequences of a complete open reading frame (ORF) (Matthijnssens et al., 2008a). In this system, 80% VP4 and VP7 nucleotide sequence identities are the cut-off values for the classification of P and G genotypes. To date, 30 P-genotypes (P[1]-P[28] and P[30]-P[31]) and 21 G-genotypes (G1-G20 and G22) have been described in literature (Matthijnssens et al., 2008a; Schumann et al., 2009; Solberg et al., 2009). The G and P genotypes are peculiarly distributed across various animal species, suggesting host species barriers and restriction. For example, G1P[8], G2P[4], G3P[8] and G4P[8] strains are globally predominant in humans (Estes, 2001). However, human rotaviruses with unusual G genotypes (e.g., G5, G6, G8, G9, G10 and G12) and P genotypes (e.g., P[3], P[6], P[9], P[11] and P[14]) have been emerging, suggesting that natural reaasortments have occurred between human and animal rotaviruses, especially in cattle and pigs (Dhama et al., 2009; Ghosh et al., 2007; Li et al., 2008; Nguyen et al., 2007; Parra et al., 2008; Rahman et al., 2007; Santos and Hoshino, 2005; Sharma et al., 2008). G9 rotavirus, of which the VP7 gene is presumably derived from a porcine rotavirus, is recognized as the emerging pathogen for humans worldwide (Hoshino et al., 2005; Phan et al., 2007b; Yang et al., 2007). Therefore, animal rotaviruses are regarded as a potential reservoir for genetic diversity of human rotaviruses.

It is notable that a number of new genotypes have recently been found in animals (Liprandi et al., 2003; Martella et al., 2006, 2007; McNeal et al., 2005; Parra et al., 2007; Rao et al., 2000; Schumann et al., 2009). Monitoring of newly emerging rotaviruses in animals is important for the control of rotavirus infection in both humans and animals. Therefore, there has been great interest in rotaviruses circulating in our environment. However, despite the fact that rotaviruses cause asymptomatic infection in calves, pigs, cats and humans (Dea et al., 1985; Debouck and Pensaert, 1983; Hoshino et al., 2003; McNulty and Logan, 1983; Mochizuki et al., 1997; Ray et al., 2007; Reynolds et al., 1985; Roger et al., 2005; Steyer et al., 2008, 2007), there is little information on rotavirus infection in healthy animals. Hence, it remains to be elucidated how asymptomatic infection with rotaviruses affects the ecology and evolution of rotaviruses. Our previous seroepidemiological study showed high prevalences of infection with rotaviruses of several genotypes in cattle (unpublished data). Thus, it is expected that surveillance of rotavirus infection in cattle will provide further information on the natural history of rotaviruses.

To understand infection cycles of rotaviruses in nature, it is necessary to characterize the viruses in subclinically infected animals. For this purpose, we examined healthy calves in Gifu Prefecture, Japan for rotavirus infection during the period from 2006 to 2007. We were able to isolate an unknown bovine group A rotavirus from the feces of healthy cattle. Genetic analysis of the strain demonstrated that VP4 and VP7 have less than 80% nucleotide identities with other established P- and G-genotypes, and therefore the Rotavirus Classification Working Group (RCWG) assigned novel P[29] and G21 genotypes (Matthijnssens et al., 2008b).

Furthermore, to clarify when G21P[29] rotavirus emerged in cattle populations, we retrospectively investigated the prevalence of G21P[29] rotaviruses in healthy calves using the fecal specimens collected in Hokkaido, Japan from 1997 to 1998. The results suggested that G21P[29] rotaviruses have been endemic in calves for at least 10 years.

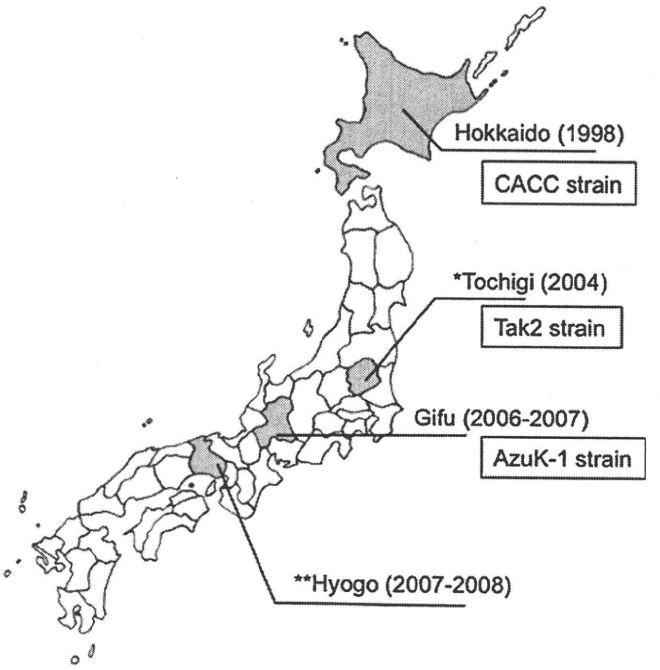

Fig. 1. Geographical distribution of G21P[29] rotaviruses in Japan. *Fukai et al. (2007); **unpublished data.

2. Materials and methods

2.1. Fecal samples

A total of 171 fecal specimens were collected from healthy calves (aged <1 year) on a beef cattle farm located in Gifu Prefecture, Japan from July 2006 to June 2007 (Fig. 1). The fecal samples were diluted with phosphate-buffered saline to 20% suspensions and clarified by centrifugation at 750 × *g* for 10 min. The supernatants were collected and stored at −80 °C until use.

In addition, we investigated 298 fecal specimens collected from eight healthy calves in Hokkaido Animal Research Center in Japan during the period from 1997 to 1998 (Fig. 1). These fecal specimens were diluted with phosphate-buffered saline to 10% suspensions and clarified by centrifugation at 750 × *g* for 10 min. The supernatants were collected and stored at −20 °C until use.

2.2. RNA extraction, reverse transcription, and nested and semi-nested PCR (RT-PCR)

Viral RNA was extracted from fecal suspensions by using a QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany). Synthesis of the cDNA was performed using a PrimeScript 1st strand cDNA Synthesis Kit (TaKaRa BIO, Shiga, Japan) with random hexanucleotides as primers. Genomic RNAs were heated at 95 °C for 5 min and immediately chilled on ice, and then reverse transcription reaction was carried out. The cDNAs were amplified by semi-nested PCR. To detect and analyze the rotavirus genes, we designed primers for amplifying and sequencing VP4 and VP7 genes (Supplementary Table 1). The cDNA was amplified by an outer PCR with the primers VP4-HeadF and VP4-1092R for VP4, resulting in a 1050-bp product, and by an inner PCR with the primers VP4-HeadF and VP4-887R, resulting in an 840-bp product. PCR was carried out with TaKaRa Ex Taq (TaKaRa BIO, Shiga, Japan) on a PC-320 PROGRAM TEMP CONTROL SYSTEM (ASTEC, Fukuoka, Japan) and TP600 TaKaRa PCR Thermal Cycler Dice Gradient (TaKaRa BIO, Shiga, Japan). Inner and outer PCRs were performed with an initial denaturation step at 95 °C for 5 min, followed by 50 cycles of 95 °C for 30 s, 50 °C for

1 min, 72 °C for 1 min and a final extension at 72 °C for 5 min. VP7 genes were partially amplified by nested PCR with the outer primer pair Beg9 and End9 (Gouvea et al., 1990) and the inner primer pair VP7-up2 and VP7-down3, producing gene amplicons of about 1000 bp and 920 bp, respectively. The PCR conditions were the same as those described for VP4.

2.3. Direct sequencing and DNA sequence analysis for P and G genotyping

The second PCR products were purified with NucleoSpin Extract II (MACHEREY-NAGEL, Duren, Gremany) and sequenced with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an ABI PRISM 3100 DNA analyzer (Applied Biosystems, Foster City, CA). The VP4-HeadF, VP4-887R, VP7-up2 and VP7-down3 PCR primers were also used as sequencing primers. The nucleotide and amino acid sequences of VP4 and VP7 genes were compared with those of the established 30 P and 21 G genotypes of rotavirus strains available in GenBank by CLUSTAL W (Tompson et al., 1994).

2.4. Virus isolation

Virus isolation was performed by a method similar to that previously reported (Fukusyo et al., 1981). Briefly, 20% fecal suspension was added to an equal volume of minimal essential medium (MEM) (Wako, Osaka, Japan) supplemented with 100 μg/ml gentamicin (GIBCO, Grand Island, NY) and 2.5 μg/ml amphotericin B (Sigma, St. Louis, MO). The suspension was treated with an equal volume of 20 μg/ml trypsin (Sigma, St. Louis, MO) for 30 min at 37 °C and inoculated onto a confluent monolayer of African green monkey kidney (MA-104) cells. After adsorption for 1 h, the monolayer was washed three times and maintained in serum-free MEM supplemented with 0.5 μg/ml trypsin, 50 μg/ml Gentamicin and 2.5 μg/ml Amphotericin B using a roller drum apparatus for 1 week. After three cycles of freeze-thawing, the suspension was clarified by centrifugion at 750 × *g* for 15 min and then inoculated into MA-104 cells. Additional passages were performed twice by the same method as that described above. The presence of rotavirus was determined by an indirect immunofluorescence assay in MA-104 cells using anti-VP6 monoclonal antibody as described previously (Minamoto et al., 1993).

2.5. Determination of 5′ and 3′ terminal sequences

To obtain the complete nucleotide sequence of the VP4 and VP7 genes of the rotavirus strain AzuK-1, we used the single-primer amplification method described previously (Ito et al., 2001). Briefly, a single amino-linked modified oligonucleotide, an adaptor primer (Supplementary Table 1), was ligated to the 3′ ends of both senses of dsRNA by using T4 RNA Ligase (TaKaRa BIO, Shiga, Japan). Following reverse transcription using P-1, a complementary to adaptor primer (Supplementary Table 1), the cDNA including the entire 5′ or 3′ terminal sequence of each segment was amplified by nested PCR using the primer pair P-1 for the first PCR or P-2 for the nested PCR and appropriate gene-specific primers, VP8-1, VP4rF2, VP7-1 and VP7-2 (Supplementary Table 1). These primers were prepared on the basis of sequence data obtained in this study. PCR products were purified and used for nucleotide sequencing as described above.

2.6. Sequencing and phylogenetic analysis

Sequences were assembled and analyzed using A plasmid Editor v1.10.4. Alignments of the nucleotide and deduced amino acid sequences of VP4 and VP7 genes of AzuK-1 strain with those of reference strains obtained from NCBI GenBank database were performed using CLUSTAL W. Phylogenetic trees were constructed by the neighbor-joining method using the MEGA version 4.0 software (Tamura et al., 2007).

Table 1
Detection of rotavirus VP4 genes in normal bovine feces by RT-nested PCR.

Year	Month	Positive/samples	Positive rate (%)
2006	July–November	0/39	0
	December	2/1	14.3
2007	January	3/12	25.0
	February	1/23	4.3
	March	1/21	4.8
	April	0/21	0
	May	1/20	5.0
	June	1/21	4.8
Total		9/171	5.3

2.7. Nucleotide sequence accession numbers

The nucleotide sequence data presented in this paper have been deposited in the GenBank database. The accession numbers of the genes encoding VP4 and VP7 of AzuK-1 strain are AB454420 and AB454421 and those of the genes encoding VP4 and VP7 of CACC strain are AB486010 and AB486011, respectively. The accession numbers for the partial VP8* and VP7 gene sequences are as follows: AB457634 (VP8*) for AzuK-3 strain, AB457635 (VP8*) and AB457636 (VP7) for AzuK-7 strain, and AB457637 (VP8*) for AzuK-9 strain.

Reference rotavirus strains and their accession numbers for the VP4 gene used in this study were as follows: NCDV (AB119636), SA11 (M23188), RRV (M18736), L26 (M58292), UK (M22306), Gottfried (M33516), OSU (X13190), Wa (L34161), AU-1 (D10970), 69M (M60600), B223 (D13394), H-2 (L04638), MDR-13 (L07886), Sun9 (AB158430), Lp14 (L11599), EDIM (AF039219), PO-13 (AB009632), L338 (D13399), Mc345 (D38054), EHP (U08424), Hg18 (AF237665), 160/01 (AF526374), 34461-4 (AY768809), TUCH (AY596189), Dhaka6 (AY773004), 134/04-15 (DQ061053), CMP034 (DQ534016), Ecu534 (EU805773), Ch-2G3 (EU486956) and Ch-661G1 (EU486962). Reference rotavirus strains and their accession numbers for the VP7 gene used in this study were as follows: KU (D16343), S2 (M11164), RRV (M21650), Gottfried (X06386), OSU (X04613), NCDV (M12394), Ch-2 (X56784), A5 (D01054), 116E (L14072), 61A (X53403), YM (M23194), L26 (M58290), L338 (D13549), CH3 (D25229), Hg18 (AF237666), EW (U08430), Ty-1 (S58166), PO-13 (D82979), Ch-1 (AB080738), Ecu534 (EU805775) and Tu-2E10 (EU486973).

3. Results

3.1. Detection of rotavirus genes from normal feces

A total of 171 fecal specimens from healthy calves were initially screened for the presence of rotavirus by detection of VP4 genes using RT-semi-nested PCR. As a result, rotavirus VP4 genes were detected in nine (5.3%) of the 171 samples from asymptomatic calves (Table 1). Seven of the nine samples were found to be positive in the first-step PCR, indicating that a considerable amount of virus was excreted in normal feces. Rotavirus genes were identified throughout a 4-month period from December 2006 to March 2007 and throughout a 2-month period from May to June 2007. The highest detection rate of rotavirus genes was in January (25.0%), followed by December (14.3%), whereas detection rates in other months were low (4.3–5.0%).

Table 2
Possible P and G genotypes of the nine detected rotavirus strains.

Strains	Year	Month	Possible P and G[a] types	
			P type	G type
AzuK-1	2006	December	P[?][b]	G?
AzuK-2		December	P[?]	G?
AzuK-3	2007	January	P[?]	G?
AzuK-4		January	P[?]	G?
AzuK-5		January	P[?]	G?
AzuK-6		February	P[?]	G(ND[c])
AzuK-7		March	P[11]	G10
AzuK-8		May	P[?]	G?
AzuK-9		June	P[11]	G(ND)

[a] VP7 genes were also amplified by RT-nested PCR and sequenced to determine possible G types.
[b] Untypeable due to low nucleotide identity to any known rotaviruses.
[c] Not determined because VP7 genes were not detected by RT-nested PCR.

3.2. P genotyping by sequence analyses of partial VP8* genes

For P genotyping of the nine strains, the VP4 genes were partially sequenced (693 bp, corresponding to nucleotides 100–786 of the RRV VP4 gene) and were compared with those of 30 established P genotype strains. Based on the nucleotide similarity of partial VP8* nucleotide sequences, the nine strains were divided into two P genotypes (Table 2). One genotype (AzuK-1, AzuK-2, AzuK-3, AzuK-4, AzuK-5, AzuK-6 and AzuK-8) was untypeable and the partial VP8* amino acid sequence was compared with those of representatives strains of the 30 established P-genotypes, resulting in very low identities (33.6–51.7%). These untypeable strains had identical sequences except for one strain (AzuK-3), which differed by two nucleotides. The other genotype (AzuK-7 and AzuK-9) was considered to be P[11] due to highest amino acid identities (96.8% and 96.3%, respectively) to bovine B223 strain belonging to P[11] genotype.

3.3. G genotyping by sequence analyses of partial VP7 genes

Similar to the P genotyping, the VP7 genes of the nine strains were partially sequenced (682 bp, corresponding to nucleotides 149–830 of the RRV VP7 gene) and were compared with those of 21 other established G genotype strains. Among the seven strains for which P genotypes were nontypeable, six strains (AzuK-1, AzuK-2, AzuK-3, AzuK-4, AzuK-5 and AzuK-8) had the same sequences of partial VP7 genes, suggesting that they belong to the same G genotype, whereas the VP7 gene of the other strain (AzuK-6) was not detected by RT-PCR (Table 2). When the partial VP7 amino acid sequences of these strains were compared to those of references strains, low degrees of identities (57.5% to 73.9%) were found. Thus, the G genotype of these six strains were also untypeable. The G genotype of AzuK-7 strain was considered to be G10 due to highest amino acid sequence identities of the partial VP7 gene with bovine rotavirus 61A strain (96.4%) belonging to G10 genotype, whereas the G genotype of AzuK-9 strain was undetermined because the VP7 gene was not detected by RT-PCR.

3.4. Isolation of a novel bovine rotavirus

One of the untypeable strains was isolated from normal feces of a 3-month-old calf. A typical cytopathic effect was observed after inoculation of the fecal suspension into MA-104 cells. The isolate was passaged twice in MA-104 cells and designated as strain AzuK-1. In an indirect immunofluorescence assay using an anti-VP6 monoclonal antibody that recognizes all group A rotaviruses (Minamoto et al., 1993), marked fluorescence was observed in MA-104 cells inoculated with AzuK-1 strain (data not shown). Furthermore, by sequencing the partial VP4 and VP7 genes of AzuK-1 strain, it was confirmed that the isolated virus genes were identical to those amplified by RT-PCR from the fecal sample. To investigate the untypeable rotavirus in detail, AzuK-1 strain was further characterized by analyzing the full lengths of VP4 and VP7 genes.

Table 3
Comparison of the nucleotide and amino acid sequence identities of the genome segment encoding protein VP4 of AzuK-1 strain with those of 30 known P genotypes strains.

Strains	Origin	P genotype	Identity with AzuK-1 strain (%)	
			Nucleotide	Amino acid
NCDV	Bovine	P[1]	65.9	65.3
SA11	Simian	P[2]	65.7	63.9
RRV	Simian	P[3]	65.2	63.4
L26	Human	P[4]	63.5	61.4
UK	Bovine	P[5]	63.9	63.5
Gottfried	Porcine	P[6]	62.8	59.4
OSU	Porcine	P[7]	65.3	63.6
Wa	Human	P[8]	64.2	60.7
AU-1	Human	P[9]	63.0	61.0
69M	Human	P[10]	64.2	63.9
B223	Bovine	P[11]	59.1	57.2
H-2	Equine	P[12]	65.9	62.5
MDR-13	Porcine	P[13]	65.8	62.3
Sun9	Bovine	P[14]	63.2	59.5
Lp14	Ovine	P[15]	64.4	63.5
EDIM	Murine	P[16]	61.7	62.9
PO-13	Pigeon	P[17]	60.8	56.4
L338	Equine	P[18]	64.9	61.3
Mc345	Human	P[19]	64.9	62.4
EHP	Murine	P[20]	62.6	61.9
Hg18	Bovine	P[21]	65.3	63.0
160/01	Lapine	P[22]	58.2[a]	49.1[a]
34461-4	Porcine	P[23]	59.9[a]	51.3[a]
TUCH	Simian	P[24]	65.0	63.0
Dhaka6	Human	P[25]	64.0	61.5
134/04-15	Porcine	P[26]	65.4	64.1
CMP034	Porcine	P[27]	64.0	61.7
Ecu534	Human	P[28]	65.0	63.4
Ch-2G3	Chicken	P[30]	61.3	55.2
Ch-661G1	Chicken	P[31]	61.5	54.7

[a] Calculation of amino acid identity was based on the VP8* region of the VP4 gene.

3.5. Sequence and phylogenetic analyses of the VP4 gene

The VP4 gene of AzuK-1 strain was found to be 2373 nucleotides in length and to code 777 amino acids. The VP4 gene of AzuK-1 strain was longer than the lengths of other rotavirus strains (mostly about 2362 nucleotides) due to several nucleotide insertions in the 3′ non-coding region (data not shown). The VP4 coding nucleotide and deduced amino acid sequences of AzuK-1 strain were compared with those of 30 established reference P genotypes (Table 3). Identity of those in VP4 was low, ranging from 59.1% to 65.9% (nucleotide level) and from 54.7% to 65.3% (amino acid level). These values were below a cut-off value of 80% nucleotide sequence identity that has been used to classify P genotypes (Matthijnssens et al., 2008a). A phylogenetic tree based on the VP4 nucleotide sequences of 30 established P genotypes strains and AzuK-1 strain showed that AzuK-1 strain was located in a new branch (Fig. 2). These results indicated that AzuK-1 strain belongs to a novel P genotype. The VP4 sequence of AzuK-1 strain was confirmed to be a new VP4 genotype: P[29] by the RCWG (Matthijnssens et al., 2008b).

3.6. Sequence and phylogenetic analyses of the VP7 gene

VP7 of AzuK-1 strain was 1062 nucleotides in length, with two in-phase open reading frames beginning at nucleotides 49 and 121 and a single UAG codon at nucleotide 1027. Compared to other G genotypes (Estes, 2001), the second open reading frame of AzuK-1

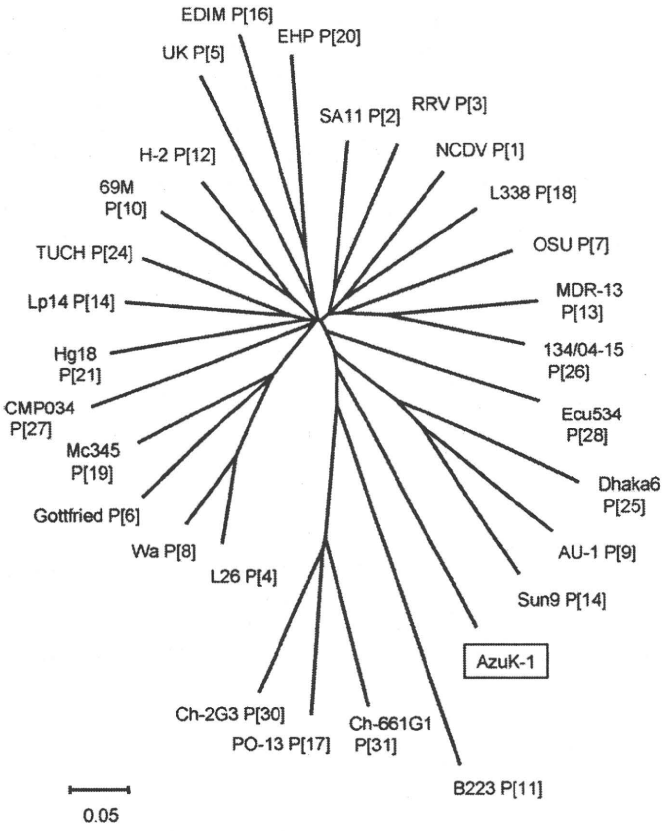

Fig. 2. Phylogenetic tree based on the VP4 nucleotide sequences of 30 established reference P genotype strains and AzuK-1 strain. The tree was generated by the neighbor-joining method using the MEGA version 4.

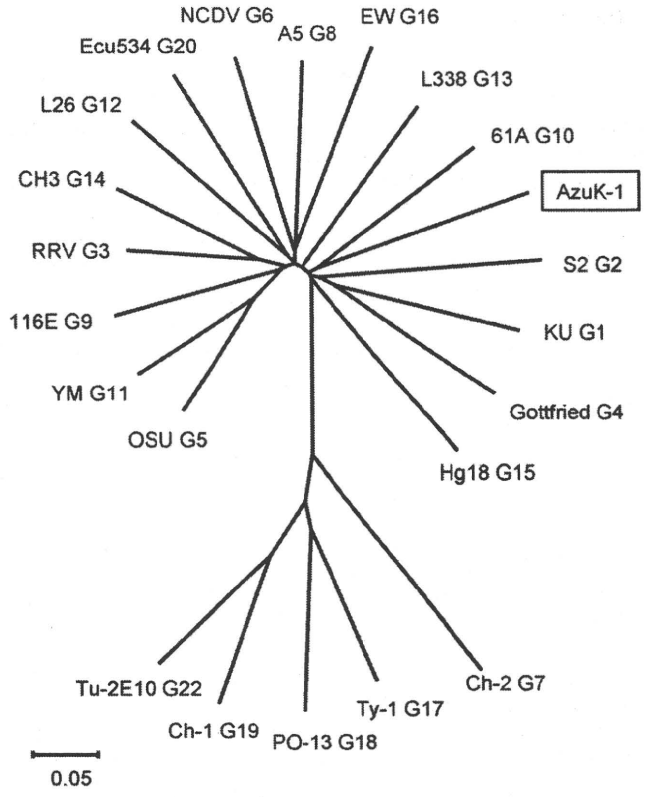

Fig. 3. Phylogenetic tree based on the VP7 nucleotide sequences of 21 established reference G genotype strains and AzuK-1 strain. The tree was generated by the neighbor-joining method using the MEGA version 4.

strain was located about 15 nucleotides upstream (data not shown). These nucleotide sequences encoded a predicted protein of 326 or 302 amino acids. The VP7 coding nucleotide and deduced amino acid sequences of AzuK-1 strain were compared with those of 21 established reference G genotypes (Table 4). VP7 of AzuK-1 strain nucleotide and amino acid sequences showed low levels of identities to those of other G genotypes, ranging from 62.4% to 73.8% and from 54.6% to 75.2%, respectively. As with the VP4 gene, these values were below a cut-off value of 80% nucleotide sequence identity that has been used to classify G genotypes (Matthijnssens et al., 2008a). A phylogenetic tree was constructed on the basis of VP7 nucleotide sequences of 21 established G genotypes strains and AzuK-1 strain (Fig. 3). In this phylogenetic tree, AzuK-1 strain was located in a new branch and was confirmed to be a new VP7 genotype: G21 by the RCWG (Matthijnssens et al., 2008b).

Table 4
Comparison of the nucleotide and amino acid sequence identities of the genome segment encoding protein VP7 of AzuK-1 strain with those of 21 known G genotypes strains.

Strains	Origin	G genotype	Identity with AzuK-1 strain (%)	
			Nucleotide	Amino acid
KU	Human	G1	73.1	75.2
S2	Human	G2	70.8	70.9
RRV	Simian	G3	72.7	74.5
Gottfried	Porcine	G4	71.9	71.2
OSU	Porcine	G5	72.6	72.7
NCDV	Bovine	G6	72.4	73.0
Ch-2	Chicken	G7	62.7	54.6
A5	Bovine	G8	70.8	71.5
116E	Human	G9	72.4	72.4
61A	Bovine	G10	73.8	73.0
YM	Porcine	G11	72.2	73.5
L26	Human	G12	72.2	73.3
L338	Equine	G13	72.9	73.0
CH3	Equine	G14	72.5	72.4
Hg18	Bovine	G15	71.5	69.6
EW	Murine	G16	71.5	72.4
Ty-1	Turkey	G17	64.6	57.7
PO-13	Pigeon	G18	62.6	54.9
Ch-1	Chicken	G19	63.5	55.2
Ecu534	Human	G20	71.4	72.7
Tu-2E10	Turkey	G22	62.4	55.5

3.7. *Retrospective study of prevalence of G21P[29] rotavirus in cattle*

G21P[29] rotavirus infection in cattle was revealed for the first time in this study. Hence, we were interested to know when genotype G21P[29] rotaviruses emerged in the cattle populations. We therefore investigated the presence of G21P[29] rotavirus in fecal samples collected from eight healthy calves in Hokkaido, Japan during the period from 1997 to 1998. Of 298 samples, six from three calves were positive by RT-semi-nested PCR using specific primers, CACCVP4-232F, VP4-189F and VP4-479R based on the VP7 and VP4 genes of AzuK-1 strain (Supplementary Table 1). Sequence analysis revealed that these six strains were genetically identical. The complete ORFs of the VP7 and VP4 genes of the representative CACC strain were determined and compared with those of AzuK-1 strain. The identities of VP4 and VP7 genes between CACC and AzuK-1 strains were 98.1% and 98.6% at the nucleotide level and 98.8% and 99.1% at the amino acid level, respectively, indicating that strain CACC also belonged to the G21P[29] genotype.

4. Discussion

In this study, we showed that rotaviruses were excreted from asymptomatic calves, especially during winter (Table 1). Rotaviruses show a seasonal pattern of infection in Japan and other countries with a temperate climate, with epidemic peaks occurring in cooler months (D'souza et al., 2007; Estes, 2001; Konno et al., 1983; Phan et al., 2007a). Thus, we cannot exclude the possibility that rotavirus excreted from subclinical calves becomes a source of infection and triggers a diarrhea outbreak. In fact, it has been reported that inoculation of a rotavirus strain isolated from a subclinically infected calf resulted in diarrhea in gnotobiotic two-day-old calves (Bridger, 1994). The fact that we detected rotaviruses mostly in first-step PCR (seven of nine samples being positive by first-step PCR) in the present study suggested that enough amount of virus for transmission to animals might be excreted in normal feces. It was also shown in some previous studies that duration of rotavirus shedding in feces and maximum yield of virus antigen in asymptomatic animals were similar to those in diarrheic animals (Archambault et al., 1990; Bridger et al., 1992a, 1992b; Hall et al., 1993). Extensive follow-up studies will be required to confirm whether asymptomatically infected calves play an important role in triggering an outbreak of rotavirus diarrhea in winter.

Analyses of partial sequences of VP4 and VP7 genes indicated that viruses detected in this study probably belong to untypeable P and G genotypes and G10P[11] (Table 2). Rotavirus strains bearing G10P[11] are common pathogens of cattle in various regions (Garaicoechea et al., 2006; Monini et al., 2008; Reidy et al., 2006; Varshney et al., 2002), including Japan (Fukai et al., 1999, 2002; Okada and Matsumoto, 2002). It has also been reported that G10P[11] strains are associated with symptomatic and asymptomatic infections in children in India (Iturriza-Gomara et al., 2004). It would be interesting to determine whether there are any genetical differences between "asymptomatic" and "symptomatic" G10P[11] strains.

We successfully isolated and sequenced the VP7 and VP4 encoding gene segments of an unknown group A rotavirus from an asymptomatic calf. This strain, AzuK-1, showed nucleotide identities below the 80% cut-off values for both VP7 and VP4, when compared to all established P- and G-genotypes, indicating that AzuK-1 possessed a new P- and G-genotype. Furthermore, our phylogenetic analyses clearly demonstrated that AzuK-1 strain was classified into novel P and G genotypes (Figs. 2 and 3). Thus, it was confirmed that AzuK-1 strain was classified into a new VP7 genotype, G21, and a new VP4 genotype, P[29], by the RCWG (Matthijnssens et al., 2008b).

The AzuK-1 strain is thought to be closely related to bovine rotavirus Tak2 strain, which was detected in Tochigi Prefecture, Japan in 2004 (Fukai et al., 2007), due to the high nucleotide identities between these two strains (99.4% in both partial VP5* and VP7 genes). From the sequence data of partical VP5* (817 bp) and VP7 (522 bp) of Tak2 strain, Tak2 strain is considered to be the same G21P[29] genotype as that of AzuK-1 strain.

It is noteworthy that AzuK-1-like rotavirus strains were also detected in other regions in Japan far from our study region, Gifu Prefecture. Tochigi Prefecture, in which Tak2 strain was detected, is about 300 km northeast of Gifu Prefecture (Fig. 1). We also detected AzuK-1-like genotypes in specimens from healthy calves in Hyogo Prefecture, which is about 200 km southwest of Gifu Prefecture (Fig. 1). Thus, it is speculated that G21P[29] rotaviruses are widely prevalent among cattle in Japan.

In addition, results of a retrospective study using fecal samples that had been collected from calves in 1997–1998 for another purpose and had been kept in a freezer indicated that G21P[29] rotaviruses were present in the cattle population for at least 10 years. Therefore, G21P[29] rotaviruses can be considered to be endemic in the cattle population over the past decade and would have escaped detection due to their low pathogenicity in cattle.

On the other hand, it is intriguing that Tak2 strain, which probably belongs to G21P[29] genotype, was detected during an outbreak of diarrhea in adult cattle (Fukai et al., 2007). This suggests that G21P[29] rotaviruses have a risk of causing epidemics of diarrhea in cattle. These findings have aroused our interest in the genetic relatedness of AzuK-1 and Tak2 strains. However, genetic information on Tak2 strain is limited. Further analyses of all 11 genome segments and pathogenicity of G21P[29] rotaviruses are needed to evaluate the risk of epidemics of diarrhea in cattle.

In this study, we found that shedding of rotaviruses in healthy calves in Japan occurred in winter and we isolated a new rotavirus, G21P[29]. In addition, this study suggested that calves may have frequent subclinical infections with rotaviruses. Considering that frequent genomic reassortment among different rotavirus genotypes was accelerated by mixed infection, the calves may serve as a reservoir for viral reassortment and evolution of novel rotavirus strains. Therefore, to fully elucidate how rotaviruses in subclinically infected animals are involved in the viral infection cycle in nature, additional analyses of genes other than VP4 and VP7 will be needed. Monitoring rotaviruses that circulate in our environment will be important for the control of outbreaks in both humans and animals.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2009.05.005.

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Ⅳ. 平成22年度研究報告書

厚生労働省科学研究費補助金（食品の安心・安全確保推進研究事業）
総括研究報告書

食肉食鳥衛生検査における家禽・家畜等のウイルス性疾病検査に関する研究

研究代表者：棚林　清　国立感染症研究所獣医科学部　第三室長

研究要旨：食用に供される家禽や家畜などの食鳥・食肉検査でウイルス性疾病については手技の煩雑性などからほとんど実施されていない。本研究では検査所の協力を得ながら実施可能な検査方法の開発・改良・検証を行いウイルス学的検査体制整備のためのマニュアル案や技術的基盤を提供すること、また、ウイルス疾病の実態調査を実施することも目的として平成22年度は以下の成果が得られた。

家畜のウイルス性疾病の検査として、動物臓器の乳剤化からPCR法でのウイルス核酸を検出するまでのマニュアルを策定・改良し、食肉処理場で採材した120頭分の豚扁桃サンプルで10種類のウイルスを調査した。その結果、ブタサーコウイルス2型が78%(93/120)、ブタパルボウイルスが54%(65/120)検出されたが、E型肝炎ウイルスを含む他の8種のウイルス遺伝子は全く検出されず、以前の結果と類似し、本マニュアルで安定した検査結果が得られると考えられた。

牛のウイルス性疾病検査のモデルとした牛白血病ウイルス（BLV）ゲノムのPCRによる検出法の簡略化と標準化を目指し、新たな耐熱性DNAポリメラーゼを試用するとともに陽性対照DNAを調製した。その結果、検査時間の大幅な短縮ならびに高感度化に成功し、本改良法を基に4か所の協力食肉衛生検査所で試行を実施した。また、BLVのTax遺伝子を解析し国内の腫瘍由来BLVはPro233型とLeu233型の2系統に分けられ、系統進化解析で野生型のBLVはLeu233型であると考えられた。さらに白血病診断時のウシの年齢との関連を調べたところ、Pro233型Taxを有する変異型BLV感染牛では発症が遅くなるものと考えられた。

高病原性鳥インフルエンザ(HPAI)と症状が類似するニューカッスル病(ND)迅速検査法として開発したRT-LAMP法はclassおよびgenotypeに関わらず幅広いNDV遺伝子が検出可能であること、ND罹患鶏からのウイルス遺伝子検出が可能であることが明らかとなった。また、HPAI確定検査のための検体輸送培地、温度、糞便混入の影響を調べたところ市販品を含め輸送の際の冷蔵管理が重要であることがわかった。

開発改良中の網羅的病原体検出マイクロアレイのプローブを応用して、病原体ゲノム検出法として安価で汎用的なマイクロプレートハイブリダイゼーション法の開発を試みインフルエンザウイルスゲノムの10^4コピーを検出できた。

家畜におけるA群ロタウイルスのリスクを把握するために、と畜場に搬入された健康豚177例を調査し9.6%から人や豚に病原性を示すタイプを含めたウイルス遺伝子の排泄を確認した。さらに、混合感染や分節遺伝子交換（リアソートメント）も観察され、直接的なリスクだけでなく、遺伝子の供給源としての間接的なリスクも示唆された。野外サンプルに直接適応できるロタウイルスの全分節遺伝子を解析する簡便法に改良を加え、前年度の検出率54.5%から81.8%と改善に成功し疫学的調査への応用が期待される。

分担研究者

岡崎　克則　　北海道医療大学薬学部・教授
池田　秀利　　日本生命科学大学獣医学部・教授
伊藤　壽啓　　鳥取大学農学部・教授
杉山　誠　　岐阜大学応用生物科学部・教授

A．研究目的

と畜検査及び食鳥検査は、望診、触診等を基本とした検査実施要領に基づき行われている。異常を発見した場合には、必要に応じてさらに精密な検査を行うこととしているがウイルスの分離同定などのウイルス学的検査は煩雑で時間を要することから検査所での実施は困難でありほとんど実施されていない。本研究では、食肉などの更なる安全確保のために検査体制整備のための技術的基盤を提供することを最終目的としている。

22年度はウイルスゲノムをPCRやRT-PCR法などで検出する検査法のさらなる改良や食肉衛生検査所での試行や策定マニュアルに沿った検査の検証を目的に、豚においては多数の検体で10種類のウイルス検査を実施した。また、牛については牛白血病ウイルス（BLV）検査PCR法の簡便化と標準化のための陽性対照DNAの作出を実施するとともに、改良したPCR法を用いて協力食肉衛生検査所で発見された牛白血病疑い検体への応用を試みることとした。さらに国内BLVの詳細な分子疫学的解析と白血病発症機構の解明のためのBLV Tax遺伝子の詳細な解析を実施した。

食鳥処理場で高病原性鳥インフルエンザ（HPAI）が疑われた場合は簡易迅速診断キットによる検査を実施することとなっており、希望地方自治体への簡易キットの配布と試用を依頼した。また、確定診断のための市販品を含めた検体輸送用培地の種類、輸送温度、検体混入糞便の各種インフウレンザウイルスの安定性に及ぼす影響を調べた。また、開発した鑑別診断が必要になるニューカッスル病ウイルス（NDV）のRT-LAMP法についての各種NDV株での反応性を検証することを目的とした。

さらに、これまで開発改良してきた網羅的病原体マイクロアレイ法に使用されているプローブを利用した安価で汎用性のあるマイクロプレートハイブリダイゼーション法の開発を試みた。

ヒトを含め多種類の動物に胃腸炎を起こすA群ロタウイルスについて健康豚における感染状況調査並びに検出ウイルスゲノムの分子疫学的解析および簡便な全分節遺伝子解析法の改良を行うことを目的とした。

B．研究方法

1．豚に感染するウイルスの検査法の検討：昨年度まで検討した臓器の乳剤化、ウイルス核酸の抽出、PCRによるウイルス遺伝子の検出至るまでのマニュアルを策定するとともに、食肉処理場及び食肉衛生検査所の協力により新たに得られた豚120頭分の扁桃材料について7種のRNAウイルス（豚繁殖・呼吸障害症候群ウイルス（PRRSV）、日本脳炎ウイルス（JEV）、豚流行性下痢ウイルス（PEDV）、豚ロタウイルス（PoRV-A）、伝染性胃腸炎ウイルス（TGEV）、ゲタウイルス（GETV）、E型肝炎ウイルス（HEV））と、3種のDNAウイルス（豚サーコウイルス2型（PCV2）、オーエウキーウイルス（SuHV-1）、豚パルボウイルス（PPV））について検出を試み2年前に同一地域での検体を調べた結果と比較検討し、マニュアルの有効性を確認した。また、検出されたPCV2やPPV遺伝子の解析を行った。

2．牛に感染するウイルスの検査法の検討：OIEのマニュアルに準拠したPCR法によって腫瘍検体からのBLV-*env*遺伝子検出法の簡略化ならびに標準化を目指し、新たな耐熱性DNAポリメラーゼ Phire Animal Tissue Direct PCR kit (FINNZYMES)を試用するとともに陽性対照DNAを調製した。本改良法を用いて協力食肉衛生検査所4か所で牛白血病（疑い）の腫瘍検体でのBLV-*env*遺伝子PCR検査を試行した。

さらに。国内各地の食肉衛生検査所の協力によ

り提供された腫瘍組織から得られたBLVのTax遺伝子の塩基配列を解読し系統進化解析を行うとともに、白血病診断時の年齢とTax遺伝子型との関連性を調べた。

3．鳥インフルエンザの検査手法の検討：昨年度までに開発したHPAIとの鑑別診断が必要となるND検査のためのRT-LAMP法について、新たに2つのgenotypeのNDV3株に対する反応性の確認、強毒型NDVを実験感染させた鶏の気管およびクロアカスワブからの検出、およびゲノムRNAの抽出法として迅速簡便法(Quick Extract RNA Extraction Kit, EPICENTER Biotechnologies社)を検討した。

また、HPAI疑い検体のウイルス分離やRT-PCR検査のために必要な輸送培地、温度、糞便混入の影響についてH5亜型ウイルスおよびヒト由来ウイルス株や野生カモ由来H1亜型株を用いて安定性を調べた。

簡易検査キットを自治体担当部局経由で食鳥検査所に配布し試用してもらった。

4．DNAプローブを用いた簡便ウイルス検出法の検討：これまでに開発した網羅的病原体検出マイクロアレイ用プローブを応用してより安価で汎用性のあるマイクロプレートハイブリダイゼーション法の基礎条件の検討を行い、インフルエンザウイルスゲノムの検出を試みた。

5．食用に供される豚におけるA群ロタウイルスの感染状況調査と遺伝子解析法の確立：2009年5月に群馬県及び2010年2月に名古屋市食肉衛生検査所に搬入された健康豚それぞれ87例及び90例の計177例より採材された豚糞便サンプルについて、RT-PCR法によりA群ロタウイルスVP4遺伝子の検出を行った。陽性検体についてはVP4及びVP7遺伝子の塩基配列解析によりP及びG遺伝子型を決定した。さらに、簡便な分節遺伝子解析法であるRT-PCR法に改良を加え、RT-semi-nested PCR法の確立を行い、ロタウイルス遺伝子陽性の牛糞便20%乳剤11例を用いて、VP4、VP7、NSP2、NSP3、NSP4及びNSP5の6つの分節を対象に簡便法を試みた。

C．研究結果

1．豚に感染するウイルスの検査法の検討：

2008年及び2010年に採材された各120頭の扁桃サンプルについて、10種の主要な豚ウイルスの存在を調査した。2008年はPCV2が84%(101/120)、PPVが57%(68/120)検出され、2010年はそれぞれ78%(93/120)、54%(65/120)の検出率であり、他の8種のウイルス遺伝子は両年とも全く検出されなかった。両年の検査結果はよく似ていた。

PCV2は遺伝子多型が多いことが知られているが、今回も多様な配列が見られ、最も多く(16/22)検出されたのは、Takahagiら(JSVS 70:603-606, 2008)の遺伝子型分類によるPCV2-2E型に近縁なウイルス遺伝子であった。簡便な遺伝子型特異的なPCR法で解析した結果PCV2a型だけが陽性個体は63%、PCV2b型12%、両方陽性は3%とPCV2a型が圧倒的に多かった。一方、PPVは全く遺伝子多型が見られなかった。

2．牛に感染するウイルスの検査法の検討：新たなインヒビター耐熱性DNAポリメラーゼPhire Animal Tissue Direct PCR kit (FINNZYMES)を用いることで、従来の1晩を要した精製DNA抽出のステップを簡略でき、検出感度も出発材料換算で少なくとも5倍の感度を有していた。また、使用するPCR用プライマーとよく反応しPCR産物が本来の目的サイズ(440bp)と区別できる陽性対照DNA(986bp)を作出することができた。

栃木県県北、茨城県県西、群馬県、静岡県東部各食肉衛生検査所の協力で添付資料に示すプロトコールに従い改良BLV-env検出PCRを試行した。牛白血病（疑い）の腫瘍が認められた93頭中85頭（91.4%）がPCR陽性あった。また、腫瘍以外の検体でPCRを実施したところ5頭中15

頭、判定不能が4頭みられた。腫瘍検体の陽性率はこれまでの 93.5%との結果とほぼ同等であった。

さらに国内各地の食肉衛生検査所の協力により提供された腫瘍組織46検体から得られたBLVの Tax 遺伝子の塩基配列を解読しアミノ酸配列を比較したところ、233番アミノ酸の違いによって国内の腫瘍由来 BLV は Pro233 型あるいは Leu233 型の2系統に分けられた。系統進化解析の結果、Pro233型BLVは全て一つのクラスターに属していたことから、野生型のBLVはLeu233型であると考えられた。Taxの233番アミノ酸と白血病診断時のウシの年齢との関連を調べたところ、Leu233型ウイルス感染牛32頭の平均月齢は55.3±5.2ヶ月であったのに対し、Pro233型ウイルス感染牛 14 頭のそれは 97.1±11.3 ヶ月と有意に高かった (p=0.00035)。

3．鳥インフルエンザの検査手法の検討：昨年までに開発した NDV-RT-LAMP 法について、新たに Genotype ⅦおよびⅧの ND ウイルスの検出が可能であることが確認された。さらに、実験的な感染ニワトリで感染後1日目から死亡（感染後5〜6日）まで、全ての気管スワブからウイルス遺伝子が検出可能であった。一方、クロアカスワブからの遺伝子検出は不安定であった。また、迅速簡便なゲノム RNA 抽出法を用いた場合は、従来のスピンカラム(Viral RNA Mini Kit, キアゲン社)比べ不安定且つ感度は劣るものの、迅速簡便法でも ND ウイルス遺伝子の検出は可能であった。

検査所でのHPAI疑い簡易検査後の確認検査のための検体輸送用培地（生理食塩水、組織培養用培地 (M-MEM) および市販の BD ユニバーサルバイラルトランスポート検体輸送用培地）での H5 亜型ウイルスおよびヒト及び野鳥由来 H1 亜型ウイルスの安定性を調べたところ 4℃では 21 日間でも感染価の低下は小さかったが 20℃保存やクロアカスワブなどの実際の検体に含まれる糞便の添加により生残性の低下がみられた。

インフルエンザ簡易キットを 16 自治体で 274 検体に試用したところ特段の問題点はなかった。

4．DNA プローブを用いた簡便ウイルス検出法の検討：これまでに開発した網羅的病原体検出マイクロアレイ用プローブを応用してマイクロプレートハイブリダイゼーション法の条件を検討したところオリゴDNAプローブが50merで、ハイブリに用いる反応液は40%ホルムアミドを含む5x SSC が最適であることが明らかとなった。また、オリゴDNAプローブの検出感度の検討をおこなった結果、インフルエンザウイルスゲノム（Narita 株）10^4コピーでも検出することができた。

5．食用に供される豚におけるA群ロタウイルスの感染状況調査と遺伝子解析法の確立：と畜場に搬入された177例中17例（9.6%）の豚糞便からVP4遺伝子が検出でき、地域に関係なく健常な豚で感染量として十分なA群ロタウイルスを排泄していることが確認された。VP4及びVP7遺伝子の解析から、G及びP遺伝子型の組み合わせが9通りあった。また、牛で検出されるG6遺伝子型に極めて近縁な同遺伝子型、人ロタウイルスに近縁なG1遺伝子型のA群ロタウイルスも健常豚より検出された。また、異なった遺伝子型ウイルスの混合感染が確認でき遺伝子分節の組換え（リアソートメント）を示すサンプルも複数株見出された。

全分節遺伝子の簡便解析法について改良を行った結果、従来RT-PCR法で延べ66遺伝子中36（54.5%）が増幅でき、6つの全ての遺伝子が増幅できたのは11例中4例であったのに対し、改良法では66遺伝子中56（84.5%）の増幅が可能で、11例中5例で6つの遺伝子セットを増幅することができ、これらの塩基配列から遺伝子型を決定することができた。VP7遺伝子分節は、全てのサンプルで増幅可能となり、少なくとも主要な遺伝子型であるP及びG遺伝子型の決定は可能となった。

D．考察

家畜に感染するウイルスの検査法については、食肉処理場や食肉衛生検査所でも実施可能なウイルス遺伝子検出法をマニュアル化し改良してきた。今年度は作成したマニュアルに従って実際に豚材料について 10 種類のウイルスについて（RT）-PCR 検査を実施し、2008 年の調査結果を比較すると、この地域の養豚場で常在している豚ウイルスに2年間で大きな変化がないと推定され、策定したマニュアルで安定した結果が得られると考えられる。今回のマニュアルは（RT－）PCR による豚の主要ウイルスを簡便に特定する手法であるが、PCR プライマーの変更による PCV2 の遺伝型の判別に今年度利用可能であったことから他のウイルスの検査にも容易に応用可能であると考えられる。

今年度調査したウイルスの中で、人獣共通感染症病原体は E 型肝炎ウイルスだけであるが過去報告では、豚レバーの 1.9%（7/363）でウイルス遺伝子が検出されているが、今年度と 2008 年の計 240 頭の調査で陽性はなく検査対象組織や技術的な検出感度を精査する必要がある。今後、家畜からヒトに感染する新興感染症の新たなチェック体制の強化が必要となる場合には本研究で作成した、簡便なウイルス検査法マニュアルが参考になると考えられる。

牛に感染するウイルスの検査法開発として牛白血病ウイルス(BLV)検出 PCR 法をモデルとして改良を行ってきた。今年度はインヒビター耐性の耐熱性 DNA ポリメラーゼ Phire Animal Tissue Direct PCR kit (FINNZYMES)を使用することによりこれまでの核酸抽出ステップを大幅に簡略化でき、さらに検出の感度も良くすることができた。さらに、陽性検体での DNA バンドと異なるサイズの陽性対照 DNA を作出したことで交差汚染をモニターでき検査精度の向上に有用と考えられた。

本改良法で食肉衛生検査所での検査も可能と考えられたことより協力検査所で牛白血病（疑い）腫瘍検体の PCR を試行した。使用する器具の違いなどがあったが過去の検出率とほぼ同等の検出率であり、牛腫瘍検体の BLV 確認には検査所レベルでも可能と考えられた。しかし、目視検査で腫瘍が見られない検体で本 PCR を行うと 36%程度で PCR 陽性または判定不能の結果となった。これらの中には他の BLV－PCR 検査法で陽性となったものや抗体陽性となったものが多くあり、現在の BLV 高感染率の状況で腫瘍が見られない牛の発病判定には適用できないものと考えられる。

さらに BLV 感染と発症機構の解明のために BLV の転写活性因子である Tax の遺伝子を解析し、233 番アミノ酸の違い（Leu と Pro）があることが分かり 233Pro のウイルスでは発症が遅れることと関連していた。Env の RFLPIII 型で発症が遅れるとの昨年度の結果との関連はさらに解析が必要と考えられる。これらの変異部位を容易に検出できれば BLV 感染牛の予後診断に有効かも知れない。

高病原性鳥インフルエンザの検査においては同様の症状を示すニューカッスル病を鑑別する必要がある。本研究で開発した RT-LAMP は新たに 2 つの genotype の 3 株でも検出可能で、幅広い遺伝子型の NDV 検出に有効であることを示している。また、NDV を実験感染した鶏からのウイルス遺伝子検出も可能であったが、クロアカスワブを材料とした場合、ウイルス感染価が高くても遺伝子検出が陰性となることもあり、反応が不安定であった。これはクロアカスワブ中に RT-LAMP の反応を阻害する物質が含まれているためと考えられ気管ぬぐいを選択するべきであると考えられた。さらに、核酸精製カラムを使わない、迅速簡便 RNA 抽出法は試験機器の揃わない診断現場でも利用可能で、且つ迅速であり NDV 遺伝子の検出は可能であったが、反応が安定しない、反応液に RNA 抽出液の原液を添加すると反応が阻害される等の問題が認められた。今後、反応の安定性に関してより良い条件検討を行い、診断現場で実際に使用できるようにする必要がある。