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Detection and differentiation of Aino and Akabane Simbu serogroup bunyaviruses by nested polymerase chain reaction

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Summary. Reverse transcription-polymerase chain reaction (RT-PCR) and nested PCR were developed to detect and differentiate Aino (AINO) and Akabane (AKA) virus S RNA. Two pairs of AINO- and AKA-specific primers for nested PCR were synthesized and examined for their capacity to amplify PCR products using 7 Simbu serogroup viruses isolated in Japan and Australia. RT- and nested PCR using AKA-specific primers amplified cDNA from Tinaroo virus RNA as well as homologous RNA. Nested PCR products were differentiated by *Hph* I and *Bst* EII digestion. Peaton (PEA) virus S cDNA was also amplified using AINO-specific primers. The nested PCR products of PEA virus were not digested by 3 restriction enzymes (*Ava* II, *Eco* RI and *Hae* II), whereas those of AINO virus were digested as expected. Using this technique, AINO and AKA viruses were detected at concentrations as low as 10^{-3} plaque-forming units (PFU) and 10^{-5} PFU, respectively, in a supernatant of virus-infected cells. It was possible to detect AINO and AKA genome from various tissues of experimentally infected mice, and also the AKA nested PCR products from serum samples from sentinel cattle naturally infected with AKA virus. The present nested PCR appears a simple, rapid and valuable method for diagnosing AINO and AKA infection.

Introduction

The *Bunyavirus* genus consists of 18 serogroups, including about 170 viruses, and is the largest virus group which infects vertebrates [13]. The Simbu serogroup includes 24 antigenically related viruses present throughout the world. Whereas only 2 Simbu serogroup bunyaviruses, Aino (AINO) and Akabane (AKA) viruses, have been isolated in Japan so far, 7 Simbu serogroup viruses including AINO

and AKA viruses have been isolated in Australia. Serological surveys suggest that Douglas (DOU), Peaton (PEA) and Tinaroo (TIN) viruses may also be present in Japan (Akashi et al., unpubl. data).

AKA virus causes epizootics of congenital deformities referred to as the arthrogryposis-hydranencephaly (AH) syndrome in cattle, sheep and goats. Epizootics of this syndrome have been reported in Australia, Japan and other countries [9]. Whereas AINO virus has been attributed with causing developmental defects based on serological evidence from calves with congenital defects similar to the AH syndrome [14], the virus has not been isolated from such cases, nor has the disease been reproduced by experimental infection. AINO virus appears to produce disease much less frequently than AKA virus. However, during autumn 1995 to spring 1996, a large outbreak of AINO virus infection occurred in the southern and western parts of Japan with over 2,000 abnormal calves delivered. The reason for the occurrence of such a large outbreak is still unknown. However, it is very important to identify the causative agent of calf deformities quickly so that control measures can be developed.

The diagnosis of AINO and AKA diseases is mostly based on the detection of antibodies in precolostral sera from calves with deformities. However, for the diagnosis of very recent infection, before the development of antibodies, it is necessary to detect virus in tissues of aborted fetuses and perhaps in the blood of infected dams. Based on the sequences of AINO and AKA virus S RNA species determined previously [2, 4], we were able to develop specific primers for the detection and differentiation of these viruses by reverse transcription-polymerase chain reaction (RT-PCR) and nested PCR in virus-infected samples.

Materials and methods

Viruses and cells

The OBE-1 strain of AKA virus [11] and JaNAr28 strain of AINO virus [17] were used as the source of RNA. To determine the specificity of the reaction, 5 other Simbu serogroup viruses were usesd as controls. The CSIRO 150 strain of DOU virus [16], CSIRO 110 strain of PEA virus [16] and CSIRO 153 strain of TIN virus [16] were kindly supplied by Dr. T. D. St. George, CSIRO, Division of Animal Health, Australia. The Ch16129 strain of Facey's Paddock (FP) virus [7] and CSIRO 1 strain of Thmiri (THI) virus [15] were kindly supplied by Mrs. L. Melville, Berrimah Agricultural Research Centre, Northern Territory, Australia. Each virus was grown once to three times in monolayers of HmLu-1 cells and plaque-cloned once subsequent to their arrival at this laboratory. Methods for virus growth and purification are presented elsewhere [1].

Seven viruses known to cause abortion in cattle in Japan, No. 12 strain of bovine viral diarrhea virus, BN-1 strain of bovine parainfluenza virus 3, 758 strain of bovine herpesvirus 1, CSIRO 154 strain of bluetongue virus 21, K-47 strain of Kasba virus (*Reoviridae*, genus *Orbivirus*, Palyam virus group), BF1 strain of bovine enterovirus and BF15 strain of bovine parvovirus were used for specificity tests.

RNA and DNA extraction

Each virion RNA and DNA was extracted from virus-infected culture fluid, containing 5×10^6 PFU for Simbu serogroup viruses, using RNA STAT-50 LS (TEL-TEST, USA) and SepaGene

Table 1. Oligonucleotide primers for Akabane and Aino virus RT-PCR

Primers	Sequence	Nucleotide positions [a]
First round for Aino virus		
AISF132	5′ CCC AAC TCA ATT TCG ATA CC 3′	132-151
AISR780	5′ TTT GGA ACA CCA TAC TGG GG 3′	780-761
First round for Akabane virus		
AKSF19	5′ TAA CTA CGC ATT GCA ATG GC3′	19-38
AKSR740	5′ TAA GCT TAG ATC TGG ATA CC 3′	740-721
Nested for Aino virus		
AISF313	5′ CCA TCG TCT CTC AGG ATA TC 3′	313-332
AISR657	5′ ACA GCA TTG AAG GCT GCA CG 3′	657-638
Nested for Akabane virus		
AKSF177	5′ GAA GGC CAA GAT GGT CTT AC 3′	177-196
AKSR407	5′ GGC ATC ACA ATT GTG GCA GC 3′	407-388

[a]Numbered from the 3′ end of viral S RNA

(Sanko Jyunkaku, Japan), respectively, by the method recommended by the manufacturers. Total RNA was extracted from organs and blood samples of inoculated mice by RNAzol B(TEL-TEST, USA).

Primers

According to the S RNA sequence of the JaNAr28 strain of AINO virus [2] and the OBE-1 strain of AKA virus [4], oligonucleotide primer sets were selected using the GENETYX program (Software Development, Japan) and synthesized on a Oligo 1000 DNA Synthesizer (Beckman, USA). Primer positions on the viral genome and sequences are shown in Table 1. AKSF19 and AKSR740 primers were used to determine the sequences of nucleocapsid (N) protein genes of 23 AKA field isolates in the previous study [4, 5]. The other primers were selected from RNA regions showing less homology between AINO and AKA viruses.

RT-and nested PCR

RT-PCR was conducted using the GeneAmp RNA PCR kit (Perkin-Elmer Cetus, USA). One microliter of RNA was mixed with random hexamer in 1X PCR buffer containing 5 mM $MgCl_2$, four deoxynucleotides and reverse transcriptase in a total volume of 20 μl and incubated at room temperature for 10 min, 42 °C for 15 min, 99 °C for 5 min, then 5 °C for 5 min. PCR reaction with specific primers was carried out in a final volume of 100 μl for 25 cycles at 94 °C for 30 sec, 55 °C for 30 sec and 72 °C for 1 min, followed by a 7 min incubation at 72 °C. After the first RT-PCR, the product was purified by Magic PCR Preps (Promega, USA), and then used as template DNA for nested PCR. Nested PCR was carried out as described above with *Taq* polymerase (Amersham, UK). PCR products were resolved by electrophoresis in a 2% agarose gel and visualized by ethidium bromide staining.

Restriction enzyme digestion

Nested PCR products were digested with the enzymes *Ava* II, *Bst* EII, *Eco* RI, *Hae* II and *Hph* I to confirm the identity of the products. All restriction enzyme digestions were performed as recommended by the manufacturers.

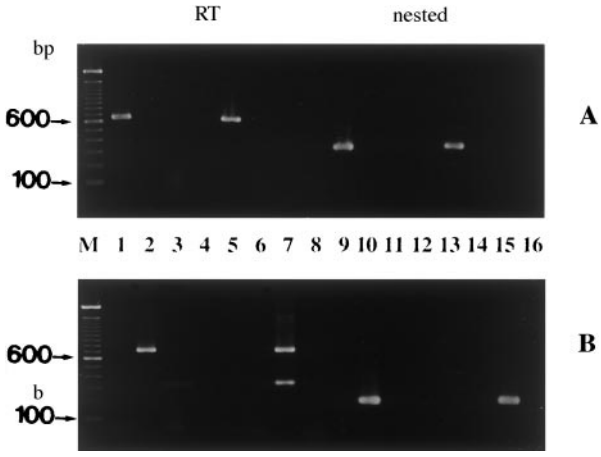

Fig. 1. Agarose gel electrophoresis of the Aino (**A**) and Akabane (**B**) specific RT- and nested PCR amplified products with 7 Simbu serogroup viruses. *M* Molecular weight marker (100 bp DNA ladder; Gibco BRL, USA); *1, 9* Aino virus; *2, 10* Akabane virus; *3, 11* Douglas virus; *4, 12* Facey's Paddock virus; *5, 13* Peaton virus; *6, 14* Thimiri virus; *7, 15* Tinaroo virus; *8, 16* cell culture fluid control. *1–8* show the results of RT-PCR, *9–16* show nested PCR

Mouse inoculation

AINO and AKA viruses were passaged once in suckling mice via the intracerebral (ic) route. Infected mouse brain suspensions containing $10^{5.5}$ $TCID_{50}$/ml served as the inoculum. Three 6-week-old mice of the BALB/c strain were inoculated by the ic route with 0.1 ml or intraperitoneal (ip) route with 0.2 ml of each virus. The inoculated mice were euthanased and exsanguinated 5 days after inoculation.

Serum samples from sentinel cattle

180 sentinel cattle sero-negative to AINO and AKA viruses were selected and serum samples collected at one-month intervals. Twenty-eight cattle seroconverted to AKA virus. Serum samples collected immediately before and after sero-conversion were tested by PCR. All sera were heat inactivated at 56 °C for 30 min and stored at −20 °C until RNA extraction. RNA was extracted by RNA STAT-50 LS.

Results

RT-and nested PCR with 7 Simbu serogroup viruses using AINO- and AKA-specific primers

The results of RT- and nested PCR with 7 Simbu serogroup viruses using AINO- and AKA-specific primers are shown in Fig. 1. Using total RNA extracted from 10^5 PFU of each virus, RT-PCR and nested PCR products of expected size (720 and 230 bp, respectively) with AKA-specific primers were obtained for AKA and TIN viruses. These results were expected on the basis of their S RNA sequence homology [3]. From AKA and TIN S RNA sequences, 2 restriction endonucleases (*Hph* I and *Bst* EII) were chosen to identify nested PCR products. The AKA nested PCR product was digested only *Hph* I and the TIN product could be cut only with *Bst* EII (Fig. 2A). Similary, PEA virus yielded about 650 bp (first round) and 350 bp (second round) PCR products with AINO-specific primers. This nested PCR product of PEA virus failed to be digested by 3 restriction enzymes (*Ava* II, *Eco* RI and *Hae* II), whereas that of AINO virus was digested as expected (Fig. 2B). No PCR product was observed from the RNA sample of uninoculated cell culture fluid.

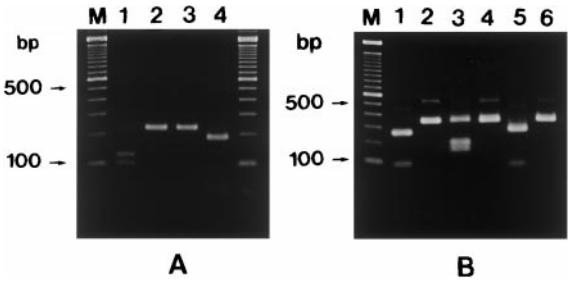

Fig. 2. Agarose gel electrophoresis of Akabane and Tinaroo (**A**), and Aino and Peaton (**B**) nested PCR products digested with several endonucleases. *M* Molecular weight marker **A** *1*, *3* Akabane virus; *2*, *4* Tinaroo virus. Products in *1* and *2* were digested with *Hph* I; *3* and *4* with *Bst* EII. **B** *1*, *3*, *5* Aino virus; *2*, *4*, *6* Peaton virus. Products in *1*, *2* were digested with *Ava* II; *3* and *4* with *Eco* RI, *5* and *6* with *Hae* II

Primer specificity was assessed using 7 viruses reported to cause abortion in cattle in Japan. No false-positive reaction was detected using the AINO- and AKA-specific primer sets (data not shown).

Sensitivity of RT-and nested PCR

To determine the sensitivity of RT- and nested PCR of AINO and AKA viruses, each RNA obtained from infectious culture fluid containing 10^5 PFU of virus was diluted 10-fold and amplified by RT-and nested PCR. About 100 PFU of AINO virus and 10 PFU of AKA virus were detected by RT-PCR, whereas 10^{-3} PFU of AINO and 10^{-5} PFU of AKA were detected by nested PCR (Fig. 3).

Detection of AINO and AKA RNA in mouse samples

AINO and AKA viruses were inoculated via the ic or ip route into mice. Brain, heart, lung, spleen, kidney and liver tissue and heparinized blood were taken from infected mice and homogenized after freezing with Eagle's minimal essential medium to make a 10% suspension. Virus titration and total RNA extraction

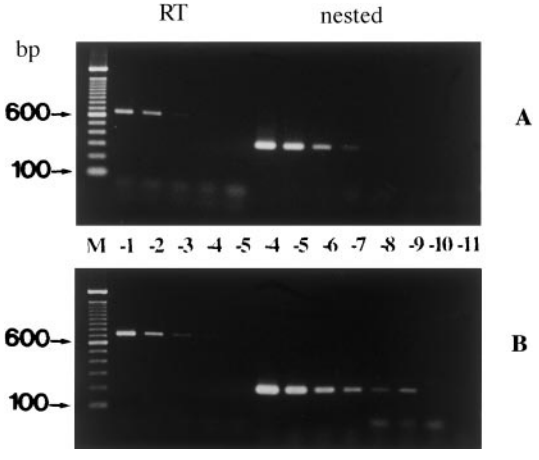

Fig. 3. Agarose gel electrophoresis of the amplified product of Aino (**A**) and Akabane (**B**) RNA. Ten-fold serial dilutions of RNA from 10^5 PFU of each virus were amplified using the specific primers by RT- and nested PCR. The figure shows from 10^{-1} to 10^{-5} dilutions for RT-PCR and from 10^{-4} to 10^{-11} for nested PCR

Table 2. Results of the RT- and nested PCR on organs from experimentally infected mice

	Brain	Heart	Lung	Spleen	Kidney	Liver	Blood
Aino-i.c.[a]							
Virus titer	5.55[b]	< 2.0[c]	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0
RT-PCR	+	−	−	−	−	−	−
nested PCR	+	−	−	+	+	+	−
Aino-i.p.[d]							
Virus titer	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0
RT-PCR	−	−	−	−	−	−	−
nested PCR	−	+	−	−	+	+	−
Akabane-i.c.							
Virus titer	5.25	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0
RT-PCR	+	−	−	−	−	−	−
nested PCR	+	+	−	−	+	−	+
Akabane-i.p.							
Virus titer	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0
RT-PCR	−	−	−	−	−	−	−
nested PCR	+	+	+	+	+	+	+

[a]Inoculated intracerebrally
[b]$\log_{10}$ $TCID_{50}$/ml
[c]Estimated detection limit ($\log_{10}$ $TCID_{50}$/ml)
[d]Inoculated intraperitonially

were conducted using these samples. Although both viruses were recovered only from the brain samples from inoculated mice by the ic route, the S RNA genome of each virus was detected in various organs of infected mice only by nested PCR (Table 2). Increasing the number of cycles of amplification failed to improve the sensitivity of RT-PCR.

Detection of AKA RNA in serum samples from sentinel cattle

Of the 28 pre-conversion sera tested, only 4 were positive by nested PCR, but not RT-PCR (data not shown). None of the post-conversion (antibody positive) sera gave a positive PCR reaction. There was no correlation between antibody titer after sero-conversion and detection of viral genome in the pre-conversion serum.

Discussion

Although only a few members of the *Bunyavirus* genus are significant pathogens in animals, 2 (AINO and AKA) bunyaviruses and Kasba virus (*Reoviridae*, genus *Orbivirus*, Palyam virus group) have been found to cause congenital deformities in cattle in Japan [8, 11, 12] and outbreaks have caused significant economic loss. Thus, monitoring for the presence of these viruses in their insect vectors

and sentinel animals is very important for disease control. Further, diagnosis of abortion often requires detection of the virus in specimens because the fetus may not yet have produced antibodies. However, diagnosis and surveillance by virus isolation is time consuming, slow and not very sensitive.

Recently, PCR has been used for the detection of Bunyamwera and California serogroup bunyaviruses from mosquito pools [10, 18]. Wasieloski et al. [18] reported that, although virus isolation was more sensitive than RT-PCR and ELISA, RT-PCR provided rapid results and was suited for mosquito samples collected in the field, which were subjected to temperature variation during transport and storage. However, insect specimens known to be infected with either AINO or AKA viruses were not available and it is very difficult to obtain aborted fetal specimens because outbreaks of AKA infection are sporadic. Therefore, we evaluated RT-and nested PCR for detecting AINO and AKA viral genes from cell culture supernatants, organs of experimentally infected mice and serum from sentinel cattle.

Although the sensitivity of RT-PCR of AINO and AKA viruses was essentially the same as reported for other bunyaviruses [6, 10], the results of nested PCR using specific primer sets demonstrate a very high sensitivity, detecting less than 10^{-3} PFU of AINO and 10^{-5} PFU of AKA viruses. This possibly may be due to the ability to detect inactivated virus and/or defective virus particles in cell culture supernatants. When tissues of experimentally infected mice were tested, PCR products of AINO and AKA viruses were detected in several organs including the brain. In comparison, viruses were only isolated from brain tissues of mice inoculated intracerebrally, suggesting that nested PCR may be more sensitive than virus isolation for AINO and AKA viruses. AKA genome was also detected in 4 serum samples from sentinel cattle in spite of heat inactivation, indicating the potential value of PCR for field specimens of inferior quality or that have been treated for other testing. Unfortunately, we were unable to obtain similar specimens from animals naturally infected with AINO virus.

The results of nested PCR with 7 Simbu serogroup viruses showed cross-reactivity between AINO and PEA, and AKA and TIN viruses. Due to the similarity of S RNA sequences of AKA and TIN viruses [3], the latter results were to be expected. Several restriction enzymes were subsequently used to identify the PCR product by restriction fragment length polymorphism (RFLP) analysis. Digestion of the products with 2 enzymes (*Hph* I and *Bst* EII) clearly distinguished between AKA and TIN viruses. Similarly, the PEA virus nested PCR products were not digested by 3 enzymes (*Ava* II, *Eco* RI and *Hae* II), whereas these enzymes digested those of AINO virus as expected [2]. Primers were selected from S segment sequences because no sequence data are currently available for other segments of any of the Simbu serogroup viruses. The specificity of RT- and nested PCR might be enhanced using primers selected from variable regions of M segments which encode the envelope glycoprotein G1 that elicits the neutralizing antibody.

In conclusion, as no reactivity of these primers was observed with either any DNA or other RNA viruses that cause abortion in cattle, a combination of this

nested PCR followed by RFLP analysis may provide a simple, rapid, and reliable means for diagnosing abortion due to AINO and AKA viruses.

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