Original Article

Monitoring of Five Bovine Arboviral Diseases Transmitted by Arthropod Vectors in Korea

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A survey was performed in Korea to monitor the prevalence of five bovine arboviruses [Akabane virus, Aino virus, Chuzan virus, bovine ephemeral fever (BEF) virus, and Ibaraki virus] in arthropod vectors, such as Culicoides species. To determine the possible applications of survey data in annual monitoring and warning systems in Korea, we examined the prevalence of bovine arboviruses in arthropod vectors using RT-PCR. To compare the sensitivity and specificity of virus detection, nested PCR was also performed in parallel for all five viruses. Using the RT-PCR, the detection limits were at least up to $10^{1.5}$, $10^{2.8}$, $10^{2.0}$, $10^{1.8}$, and $10^{4.0}$ $TCID_{50}$/ml for Akabane virus, Aino virus, Chuzan virus, BEF virus, and Ibaraki virus, respectively. When nested PCR was performed using 1 μl of PCR product, the detection limits were increased, to $10^{0.05}$, $10^{1.8}$, $10^{1.0}$, $10^{0.008}$, and $10^{2.0}$ $TCID_{50}$/ml for Akabane virus, Aino virus, Chuzan virus, BEF virus, and Ibaraki virus, respectively. Thus, nested PCR increased the sensitivity of the virus detection limit by 1~2 log. We pooled 30~40 mosquitoes in one sample. We collected 113 samples in 2006, 135 samples in 2007, and 100 samples in 2008. Among these samples, Chuzan virus and BEF virus genes were detected at a range between 0.82% and 1.19%, and Akabane virus, Aino virus, and Ibaraki virus genes were detected at less than 0.20%. These data may provide some insight into future epidemiological studies of bovine arboviral diseases in Korea.

Key Words: Arthropod vector, Bovine arbovirus, Monitoring, RT-PCR

INTRODUCTION

Bovine arboviral diseases, such as Akabane disease, Aino virus infection, Chuzan disease, bovine ephemeral fever (BEF), and Ibaraki disease, have been monitored in Korea serologically or by virus isolation. Akabane disease is

caused by the Akabane virus, which is an arthropod-borne *Bunyavirus* in the Simbu group of the family *Bunyaviridae*. Akabane disease is a major cause of epizootic congenital malformations, abortions, stillbirths, and premature births in cattle, goats, and sheep (1~4). It has been known to be endemic in Korea, Japan, and Australia, and has been suspected to be the cause of sporadic outbreaks elsewhere (1, 5~8). Aino virus also belongs to the Simbu group of the family *Bunyaviridae*, and has been known to cause the production of abnormal calves that exhibit arthrogryposis, hydranecephaly, and cerebellar hypoplasia (3, 9~11). These viruses are widely distributed in Southeast Asia, Australia, Japan, and Korea (3, 9, 12~19). Chuzan virus, a member of the Palyam serogroup, genus *Orbivirus*, and family *Reoviridae*, was first identified as a causative agent of a

cattle disease, characterized by congenital abnormalities with hydraencephaly and cerebellar hypoplasia syndrome in Japan in 1985 and 1986 (20~24). BEF, also called "three day sickness," is a viral disease caused by the BEF virus in the *Rhabdoviridae* family (18, 19, 25~29). BEF is an acute febrile infection of cattle and water buffalo, which occurs in many tropical and subtropical regions of the world (30~32). The disease shows a wide spectrum of symptoms, ranging from imperceptible clinical signs to death. The economical impact of BEF is severe because of the effects on trade, both within and between countries (26, 32~34). Ibaraki disease is caused by the Ibaraki virus (IBAV), which belongs to the genus *Orbivirus* in the family *Reoviridae* (35, 36). IBAV was first isolated from the blood of Japanese cattle in 1959 (37). Clinical signs of Ibaraki disease in cattle include fever, anorexia, and deglutitive disorder (2, 37~39).

The first recorded outbreak of Akabane disease in Korea was in 1980 (40). After the first isolation of Akabane virus, there were two major outbreaks in 1988 and 1990 (14). Aino virus infection was first unofficially reported in 1997, during an investigation of newborn deformities in calves in the southern part of Korea (17). A Chuzan disease outbreak was first reported in 1993 in the southern part of Korea (41) and there was a major outbreak in 1999 (14). BEF has a long history in Korea, as compared with the other bovine arboviral diseases. BEF virus was first isolated in Korea from diseased cattle in 1959 (42). Eight strains of IBAV were isolated in 1993 (43) and it has been believed that IBAVs existed in Korea prior to 1993.

Culicoides biting midges are the major arthropod vectors for these five viruses. Culicoides species live in subtropical to temperate regions in the world and play an important role in transmitting pathogens to humans and animals (44). Culicoides species are reported to exist in the entire geographical area of Korea (45). One research project examined Culicoides species percentages among arthropods vectors collected in specific areas in 1992 (46). These research data also showed that Culicoides species apparently disappeared for a year in Korea.

Five of these bovine arboviruses have been isolated from Culicoides biting midges or other arthropod vectors (22, 47~53). Akabane virus was isolated from a biting midge, *Culicoides oxystoma*, collected from a cowshed in Kagoshima, Japan (49). BEF viruses have also been isolated from Culicoides species on several occasions (52, 53). Chuzan virus was isolated from *Culicoides oxystoma* in Japan in 1988 (22).

In Korea, an early warning system for Japanese encephalitis (JE) has been practiced. When JE virus are detected in arthropod vectors, including Culex species, early warning signs are issued for JE. In this study, we evaluated the possibility of adapting the early warning system for bovine arboviral diseases, through the detection of arboviral genes in arthropod vectors, primarily from Culicoides species. Specifically, methods for viral genes detection from arthropod vectors were evaluated for five bovine arboviral diseases. After evaluation of the detection methods, viral genes were monitored from Culicoides species samples collected from 2006 to 2008.

MATERIALS AND METHODS

Samples

Culicoides species were collected from around the country from 2006 to 2008 in Korea. Trap lights were installed in the field to collect Culicoides species. Collected arthropods were sieved to separate Culicoides species, 1~3 mm in size, from other bigger mosquitoes and insects. Culicoides species (30~40) were pooled in one sample. These were frozen at -20℃ until used. Culicoides species were pooled (30~40) and added to 2-ml containers with ceramic beads. Samples were ground for 30 s with 1 ml of cold phosphate-buffered saline (PBS). Ground samples were centrifuged (1 min, 4℃) and supernatants were harvested for RNA extraction.

Viruses and cells

Vero cells were maintained in minimum essential medium (MEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS). Akabane virus (93FMY), Aino virus (JaNAr 28), Chuzan virus (YongAm), Ibaraki virus (Imaizumai), and bovine ephemeral fever virus (TongRae)

Table 1. The oilognucleotide primers for RT-PCR and nested PCR

Virus	PCR type	Primer name	Primer sequence	Location	Product size
Akabane virus	RT-PCR	AKAF1	TAACTACGCATTGCAATGGC	19~740	709 bp
		AKAR1	TAAGCTTAGATCTGGATACC	(S segment)	
	nested	AKAF2	GAAGGCCAAGATGGTCTTAC	177~407	230 bp
		AKAR2	GGCATCACAATTGTGGCAGC		
Aino virus	RT-PCR	AINOF1	CCCAACTCAATTTCGATACC	132~780	649 bp
		AINOR1	TTTGGAACACCATACTGGGG	(S segment)	
	nested	AINOF2	CCATCGTCTCTCAGGATATC	313~657	345 bp
		AINOR2	ACAGCATTGAAGGCTGCACG		
Chuzan virus	RT-PCR	CHUF1	TGGCTTTCTGAGGCGTTTCAGA	14~318	305 bp
		CHUR1	GGTTGCTCAATATGCCAAGCGA	(Segment 5)	
	nested	CHUF2	GATGAAGCAGCGGATGTTTT	59~292	220 bp
		CHUR2	AGCAGGTTAAGCCATACTCT		
BEF virus	RT-PCR	BEFVF1	CCTACATTGAGAGTGCCACA	1677~616	440 bp
		BEFVR1	CTTCCGTGAACAGCAACATC	(N gene)	
	nested	BEFVF2	TGAGAGTGCCACAAGGTAAA	184~511	328 bp
		BEFVR2	GCAGAGCGACCTAATCTATA		
Ibaraki virus	RT-PCR	IBAF1C	CCTAGATGTTCAATAGCAAACCTAATT	1~660	660 bp
		IBAR1	TAACATTTCGTTATAACAATAATAATT	(Segment 3)	
	nested	IBAF2	ACGTGCTTACCAACAGGAGA	40~353	314 bp
		IBAR2	TCAAGCTGTCGTGCCATATT		

were propagated at $37{℃}$ in Vero cells. Virus titers were determined in Vero cells using the Reed-Münch method (54).

RNA extraction and PCR amplification

RNA was extracted using an RNeasy mini-kit (Qiagen, Valencia, CA, USA) according to manufacturer's instruction. RNA was eluted in 40 μl distilled water. Oligonucleotide primers were commercially synthesized and are listed in Table 1. RT-PCR and nested PCR amplification primers for Akabane and Aino virus were adapted from established methods for detecting Akabane viral genes from cattle (55). These were designed to amplify S segment of Akabane and Aino virus. RT-PCR primers for Segment 5 of Chuzan virus and for N genes of BEF virus were adapted from the annual report of the NVRQS research project (14, 56). Primers for Segment 3 of IBAV were chosen from previous

Table 2. Detection limits of five bovine arboviral genes by RT-PCR and nested PCR

Virus	RT-PCR	Nested PCR
Akabane virus	$10^{1.5}$ TCID$_{50}$/ml	$10^{0.05}$ TCID$_{50}$/ml
Aino virus	$10^{2.8}$ TCID$_{50}$/ml	$10^{1.8}$ TCID$_{50}$/ml
Chuzan virus	$10^{2.0}$ TCID$_{50}$/ml	$10^{1.0}$ TCID$_{50}$/ml
BEF virus	$10^{1.8}$ TCID$_{50}$/ml	$10^{0.008}$ TCID$_{50}$/ml
Ibaraki virus	$10^{4.0}$ TCID$_{50}$/ml	$10^{2.0}$ TCID$_{50}$/ml

reports (57). RNA (5 μl) was used for RT-PCR using the One-step RT-PCR kit (Qiagen). Nested PCR primers for Chuzan virus, BEF virus, and IBAV were newly designed for this study. Viral genes were amplified using the following conditions: reverse transcription at $50{℃}$ for 30 min; after an initial denaturation at $94{℃}$ for 2 min, 35 cycles of denaturation at $94{℃}$ for 30 s; primer annealing at $58{℃}$

for 30 s; and primer extension at 68℃ for 1 min. Negative reagent controls were included in each assay. Nested amplifications used 1 μl of the RT-PCR product as a template, in a total volume of 25 μl with Taq 2× master mix (Qiagen). Nested cycling conditions were as described for RT-PCR, except that 30 cycles were used. Quality control included both positive and negative controls.

Spiking Culicoides species supernatant with virus

Supernatant from ground Culicoides species were used as diluents for virus. Aino virus and IBAV were diluted from 10^{-1} to 10^{-8} in Culicoides species supernatant, which

was confirmed negative for five arboviruses by RT-PCR and nested PCR. Total RNA was extracted from diluted virus. Extracted RNA was used for RT-PCR in parallel with RNA extracted from viruses, diluted in PBS.

RESULTS

Detection limit of five bovine arboviral genes by RT-PCR and nested PCR

It is difficult to isolate bovine arboviruses from cattle, because the duration of viremia is very short and the virus often passes unnoticed (58). To determine the feasibility of
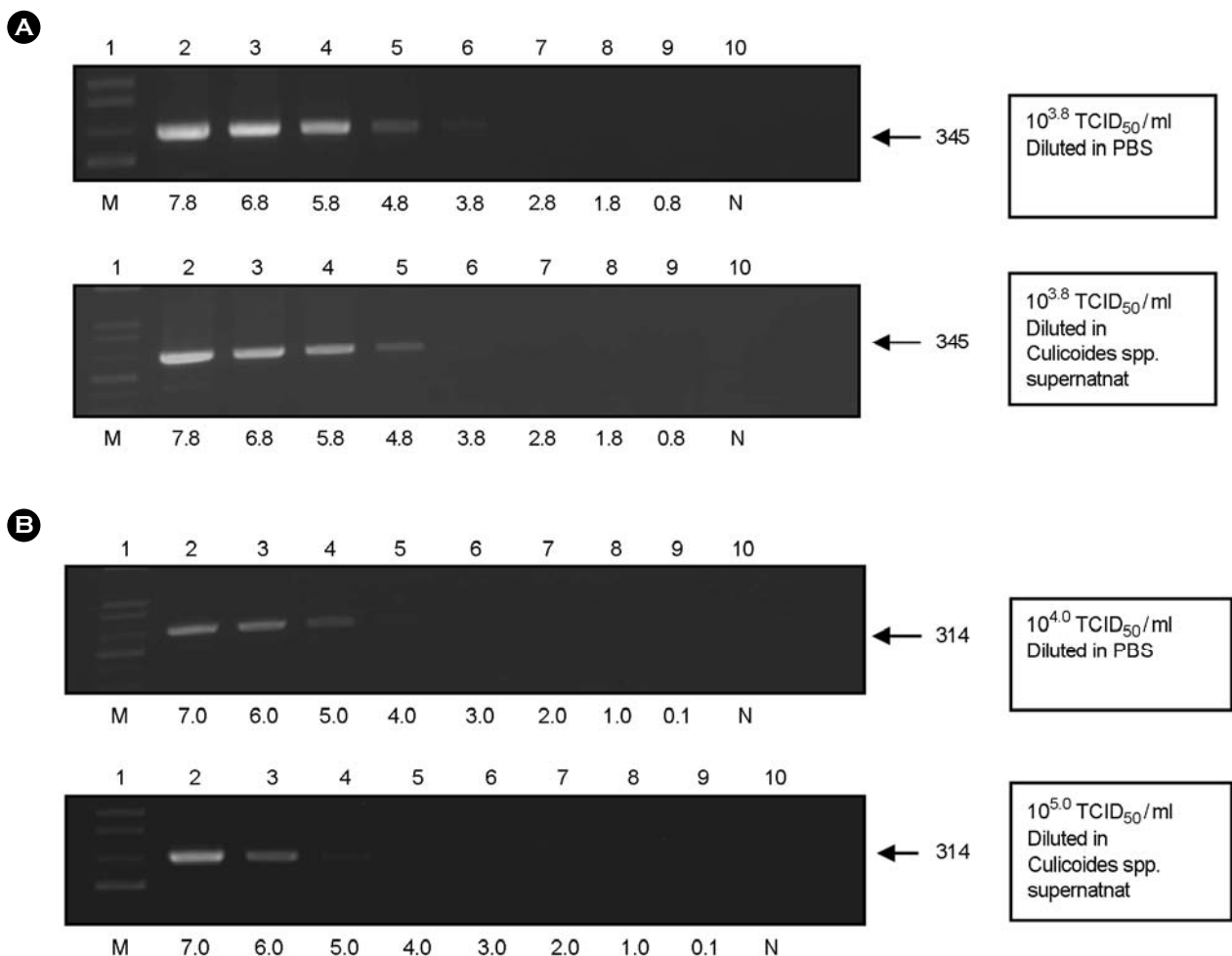

Figure 1. Detection limits determined by spiking Aino virus and Ibaraki virus into supernatant from Culicoides species. Five microliters of each PCR product from PBS-diluted virus or supernatant from Culicoides species were applied to a 1% agarose gel as follows: **(A)** Aino virus. Virus diluted in PBS (upper). 1: Marker, 2~9: diluted Aino virus, 10: negative control. Virus diluted in Culicoides species supernatant (bottom). 1: Marker, 2~9: diluted Aino virus, 10: negative control **(B)** Ibaraki virus. Virus diluted in PBS (upper). 1: Marker, 2~9: diluted Ibaraki virus, 10: negative control. Virus diluted in Culicoides species supernatant (bottom). 1: Marker, 2~9: diluted Ibaraki virus, 10: negative control.

an early warning system that detects arboviral genes in Culicoides species, RT-PCR and nested PCR methods were used. Stock virus titers were $10^{7.5}$, $10^{7.8}$, $10^{6.0}$, $10^{6.8}$, and $10^{7.0}$ TCID$_{50}$/ml for Akabane virus, Aino virus, Chuzan virus, BEF virus, and IBAV, respectively. RT-PCR results showed that the detection limit was $10^{1.5}$, $10^{2.8}$, $10^{2.0}$, $10^{1.8}$, and $10^{4.0}$ TCID$_{50}$/ml for Akabane virus, Aino virus, Chuzan virus, BEF virus, and IBAV, respectively (Table 2). When nested PCR was performed using 1 μl of PCR product, the detection limit increased, to $10^{0.05}$, $10^{1.8}$, $10^{1.0}$, $10^{0.008}$, and

$10^{2.0}$ TCID$_{50}$/ml for Akabane virus, Aino virus, Chuzan virus, BEF virus, and IBAV, respectively (Table 2). Thus, nested PCR increased the sensitivity of the virus detection by 1~2 log.

Detection limit by spiking Aino and Ibaraki virus into Culicoides species supernatant

To quantify interfering activity of RNA from Culicoides species in RT-PCR amplification, we spiked Aino and Ibaraki virus into Culicoides species supernatant, which was
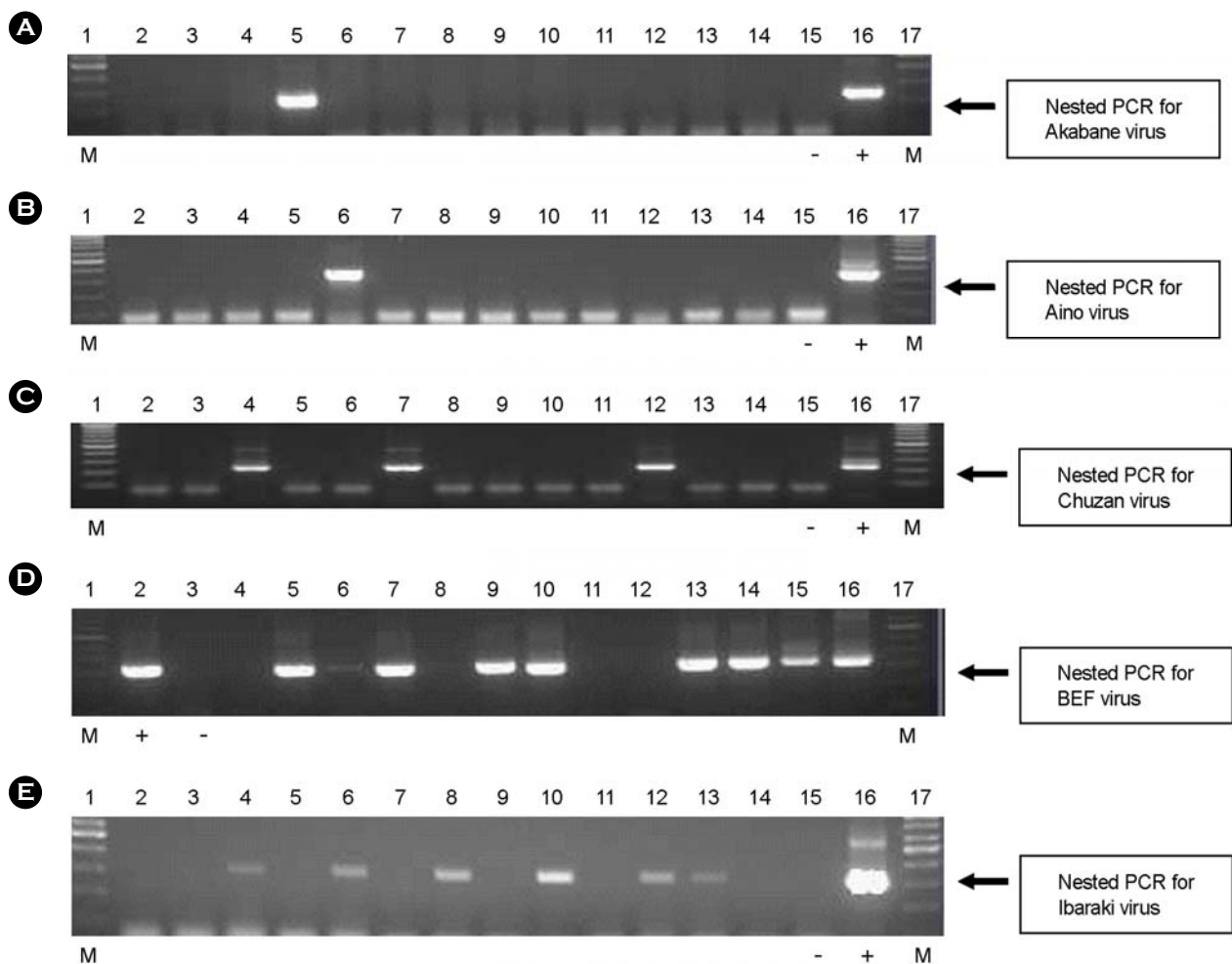

Figure 2. Prevalence of five bovine arboviral genes in arthropod vectors by nested PCR amplifications. RT-PCR, and subsequently nested PCR, were performed on RNA from pooled Culicoides species supernatant. Five microliters of the product from nested PCR were applied to a 1% agarose gel as follows: **(A)** nested PCR for Akabane virus. 1: Marker, 2~14: samples (5: Akabane virus positive sample), 15: negative control, 16: positive control, 17: Marker **(B)** nested PCR for Aino virus. 1: Marker, 2~14: samples (6: Aino virus positive sample), 15: negative control, 16: positive control, 17: Marker **(C)** nested PCR for Chuzan virus. 1: Marker, 2~14: samples (4, 7, 12: Chuzan virus positive sample), 15: negative control, 16: positive control, 17: Marker **(D)** nested PCR for BEF virus. 1: Marker, 2: positive control, 3: negative control, 4~16: samples (5, 6, 7, 9, 10, 13, 14, 15, 16: BEF virus positive sample), 17: Marker **(E)** nested PCR for Ibaraki virus. 1: Marker, 2~14: samples (4, 6, 8, 10, 12, 13: Ibaraki virus positive sample), 15: negative control, 16: positive control, 17: Marker.

Table 3. Detection limits of five bovine arboviral genes by RT-PCR and nested PCR

Year	No. of samples	No. of positive % (%)[a]				
		Akakane	Aino	Chuzan	BEF	Ibaraki
2006	113	0	4	27	25	2
		0%	3.54%	23.89%	22.12%	1.77%
		(0%)[*]	(0.10%)	(0.68%)	(0.63%)	(0.05%)
2007	135	3	4	48	78	5
		2.22%	2.96%	35.56%	57.78%	3.70%
		(0.06%)	(0.09%)	(1.02%)	(1.65%)	(0.11%)
2008	100	2	0	25	42	13
		2%	0%	25%	45%	13%
		(0.06%)	(0%)	(0.71%)	(1.20%)	(0.37%)
Total	348	5	8	100	145	20
		1.44%	2.30%	28.74%	41.67%	5.75%
		(0.04%)	(0.07%)	(0.82%)	(1.19%)	(0.16%)

[a] Percentage from sample numbers converted into numbers before pooling Culicoides species

already confirmed negative for Aino and Ibaraki viral genes by RT-PCR and nested PCR. RT-PCR results show that the detection limit for Aino virus was up to $10^{3.8}$ TCID$_{50}$/ml for both PBS-diluted virus and Culicoides species supernatant-diluted virus (Fig. 1A). In the case of IBAV, the detection limit was $10^{4.0}$ TCID$_{50}$/ml for PBS-diluted virus or $10^{5.0}$ TCID$_{50}$/ml for Culicoides species diluted virus (Fig. 1B). Because there was not much difference in RT-PCR amplification results between the two virus samples, interference from Culicoides species RNA was apparently negligible. Spiking tests for Akabane, Chuzan, and BEF virus were not performed because of the shortage of Culicoides species supernatant; however, we assumed that the results would be similar to the remaining three viruses.

Bovine arboviral genes prevalence in Culicoides species samples

We collected 113 samples in 2006, 135 samples in 2007, and 100 samples in 2008. A total of 348 samples were used for bovine arboviral genes detection (Fig. 2). The number of positive samples for Akabane, Aino, Chuzan, BEF, and Ibaraki virus using nested PCR were 0 (0%), 4 (3.54%), 27

(23.89%), 25 (22.12%), and 2 (1.77%) respectively in 2006. When the sample number was converted into 3,955 (113 samples $\times$ 30~40 Culicoides species), the prevalence percentage was 0%, 0.10%, 0.68%, 0.63%, and 0.05% for Akabane, Aino, Chuzan, BEF, and Ibaraki virus, respectively. Likewise, the number of positive samples and the prevalence percentage of samples from 2007 were 3 (2.22%, 0.06%), 4 (2.96%, 0.09%), 48 (35.56%, 1.02%), 78 (57.78%, 1.65%), and 5 (3.70%, 0.11%) for Akabane, Aino, Chuzan, BEF, and Ibaraki virus, respectively. For samples from 2008, the number of positive samples and the prevalence percentage were 2 (2%, 0.06%), 0 (0%, 0%), 25 (25%, 0.71%), 42 (45%, 1.20%), and 13 (13%, 0.37%) for Akabane, Aino, Chuzan, BEF, and Ibaraki virus, respectively (Table 3).

Homology analyses of positive PCR products

Sequencing and homology analyses were performed for Chuzan and BEF virus positive samples to rule out the possibility of contamination from the positive control, or from positive samples themselves, while performing RT-PCR or nested PCR. To rule out the possibility of cross contamination during RT-PCR or nested PCR amplification,

a portion of the positive samples were sequenced after being cloned into a pGEMT-easy vector. Three clones were selected for one positive PCR product and read twice, forwardly and reversely. It was apparent that the positive results were not from contamination of the positive control or other positive samples, because mutations or deletions were observed consistently in the same samples. These results suggest that these positive results are not from one template (data not shown).

DISCUSSION

It has been shown by virus isolation and serological surveillance that Akabane disease, Aino virus infection, Chuzan disease, BEF, and Ibaraki disease are present in Korea. In the case of Akabane disease, there were several major outbreaks in the 1980s and 1990s. Aino virus infection was first recognized in Korea due to an increased incidence of malformed calves in 1997. Although the remaining three arboviral diseases, Chuzan disease, BEF, and Ibaraki disease, have no known history of posing a major animal health threat in Korea, these five bovine arboviral diseases have been a center of attention within the cattle industry. This is primarily due to the threat of economic loss as a result of reproductive failure or decreased milk production. Recent records of Akabane disease and BEF outbreaks have resulted in six cases with 12 heads, and one case with one head affected, respectively, from 2004 to 2008. Akabane disease and BEF are notifiable diseases in Korea, whereas Aino virus infection, Chuzan disease, and Ibaraki disease are not (59). Thus, for these three diseases, if there is no special feature of a disease outbreak, such as increased numbers or severity, these may pass unnoticed.

Vaccines for Akabane disease and BEF were developed in Korea in the early 1990s and are available commercially. A trivalent inactivated vaccine for Akabane disease, Aino virus infection, and Chuzan disease was developed in 2005 and has just been released on to the market. A vaccine for Ibaraki disease was developed in 2001, but is not actively used, because there has been no known disease outbreak in Korea. To date, vaccines for Akabane disease and BEF

have been actively used, because these two vaccines are subsidized by the Korean government. Annually, 300,000 doses of BEF vaccine and 600,000 doses of Akabane disease vaccine have been provided in areas with a high risk of disease outbreak (60).

The sero-prevalence of these five arboviral diseases has been regularly monitored since 1993 to obtain information on disease status and to set up preventive measures against epidemics. If the sero-prevalence is under 30% for any of the diseases, warning signs against the disease are issued. There have been two warnings issued within the last ten years. In May 1999, a warning was issued against Akabane disease and BEF. One year later in 2000, a warning was again issued against Akabane disease, Chuzan disease, BEF, and Ibaraki disease (61). During sero-prevalence surveillance in 2008, a total of 3,077 serum samples were tested, using the serum neutralization test. Sero-positive percentages were 33.0, 19.9, 27.1, 7.2, and 28.8 for Akabane disease, Aino virus infection, Chuzan disease, and BEF, and Ibaraki disease, respectively (61). With this sero-prevalence status, if the viruses are introduced and propagated in the animal group, the damage would be high.

In this study, we evaluated the possible application of an early warning system for bovine arboviral diseases using methods for the detection of viral genes in arthropod vectors, such as Culicoides species. We may be able to apply this detection system as an early warning system against these bovine arboviral diseases, because we would be able to detect the genes of five bovine arboviruses in arthropod vectors.

Overall, the status of these five bovine arboviral diseases is non-threatening and they do not need to be dealt with as a high priority; however, with the emerging issue of global warming, there is an increasing need to re-evaluate aspects of arboviral diseases transmitted by arthropod vectors. The Korean territory has long been considered to be in the temperate zone. For the last two decades, with global warming phenomena occurring all around the world, the Korean territory is now moving into a sub-tropical zone. The rate of change within climate zones is more rapid than expected. NVRQS is planning strategies to cope with

climate changes and the expected increase in arthropod-borne animal diseases. Determining possible applications of survey data from annual monitoring and warning systems in Korea, such as detecting bovine arboviral genes in arthropod vectors, is one of the efforts for controlling diseases that are related to climate changes. Because we have shown that this detection system is able to accurately monitor arboviral diseases, it may be added to the annual national animal disease monitoring plan.

However, there are still many areas that need to be pursued. For example, the exact status of Culicoides species in Korea must be monitored. Although it has been known that there are many kinds of Culicoides species in the territory of Korea, there has not been systematic and regular surveillance for Culicoides species since 1973. There is a systematic and national level arthropod vector monitoring system in Korea, operated by the Korean Center for Disease Control. Unfortunately, not many human diseases are transmitted by Culicoides species and the monitoring program is heavily focused on arthropod vectors that transmit human diseases. Knowing the distribution of arthropod vectors that transmit animal diseases would be extremely useful for animal disease control and prevention. With a proper monitoring system, it would be possible to know the exact ecology of Culicoides species, to plan the control of diseases transmitted by these vectors, and to share the information with neighboring countries. It is likely that this information will be needed to prepare for expected increases in animal viral diseases related to global warming and climate change.

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