

## Seroepidemiological Studies of Aino Virus Infection in Korean Cattle

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Aino virus infection is characterized by abortion, stillbirth, and congenital abnormalities such as arthrogryposis-hydranencephaly syndrome in calves. In Korea, Aino virus infection was first reported in 1997 by researchers who were investigating the cause of newborn calf deformities. Given the incidence of Aino-related deformities, the need for a study of the Aino virus infection status in Korea was recognized. In this study, we investigated the nationwide seroepidemiological status of Aino virus infection. A total of 9,921 serum samples collected between 1993 and 2001, and 23,760 serum samples between 2002 and 2007 were tested using a virus neutralization assay. The seroprevalence of Aino virus was 73.1, 63.8, 44.9, 56.0, 38.5, 28.4, 18.3, 19.6, and 23.2%, respectively, between 1993 and 2001, and 43.8, 42.9, 50.7, 55.3, 31.4, and 25.4%, respectively, between 2002 and 2007. Aino virus infection does not pose a major threat to the bovine industry in Korea till now. The future prospects for Aino virus infection in cattle, however, may change with the global warming phenomena. The results of this study may serve as a basis for future epidemiological studies on Aino virus infection.

**Key Words:** Aino virus infection; Seroprevalence; Virus neutralization assay

### INTRODUCTION

Aino virus is an infectious, noncontagious, vector-borne agent that is known to cause abnormalities such as arthrogryposis, hydranencephaly, and cerebellar hypoplasia in calves (1~5). Aino virus infection is one of five major bovine arboviral diseases in Korea, alongside Akabane disease, Chuzan disease, bovine ephemeral fever, and Ibaraki disease. Among these diseases, Akabane disease, Aino virus infection, and Chuzan disease are known to cause

reproductive failure in cattle (6~8). Aino virus infection and Akabane disease are clinically indistinguishable; laboratory confirmation is the only way to differentiate the causative agents (5).

Aino virus is a member of the Simbu group in the family *Bunyaviridae*. Its genome consists of three segments of single-stranded, negative sense RNA (L [large], M [medium], and S [small]). The M RNA segment encodes two envelope glycoproteins (Gn and Gc) in a single ORF. Two polypeptides, a nucleoprotein (N) and a nonstructural protein (NSs), are encoded in overlapping reading frames in the cRNA. The L segment encodes L protein, which is an RNA-dependent RNA polymerase (9).

Aino virus was first isolated from *Culex* species in Nagasaki prefecture, Japan, in 1964 (10). Aino virus was also isolated from *Culicoides* species in Australia (11), and is now widely distributed in Southeast Asia, Australia,

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**Table 1.** Number of serum samples used in the study

Area	1993	1994	1995	1996	1997	1998	1999	2000	2001
Kyonggi	50	50	50	50	50	50	360	365	300
Kangwon	50	50	50	50	50	50	300	304	243
Chungbuk	50	50	50	50	50	50	311	346	285
Chungnam	50	50	50	50	50	50	360	367	296
Jeonbuk	50	50	50	50	50	50	300	300	214
Joennam	50	50	NT	50	50	50	240	240	200
Gyeongbuk	NT*	50	50	50	50	50	300	300	250
Gyeongnam	50	50	50	50	50	30	300	300	250
Jeju	50	50	40	50	NT <sup>a</sup>	30	60	180	150
Total	400	450	390	450	400	410	2,531	2,702	2,188

<sup>a</sup>NT: not tested

Japan, and Korea (7, 12). Seroepidemiological investigations have implicated Aino virus as the cause of epizootic and/or sporadic outbreaks of abortions, stillbirth, premature birth, and congenital abnormalities in Japanese cattle (13). In addition, Aino virus infection is a notifiable disease in Japan and has been monitored on a regular basis for quite some time (14). Active researches on Aino virus have been performed with regards to vaccine development, diagnostic material development, and viral characterization (2, 15–23).

In contrast, Aino virus infection is not well characterized and is not a notifiable disease in Korea. In fact, the first case of Aino virus infection has not yet been officially recorded in Korea. Aino virus infection was first reported in 1997 when neonatal deformities were observed in newborn calves in southern part of Korea. At that time, a nuclear power plant nearby the affected farms was blamed for the deformities. Since then, the need for a study of the Aino virus infection status in Korea was recognized. For this purpose, a total of 9,921 serum samples collected between 1993 and 2001 and 23,760 serum samples between 2002 and 2007 were tested using a virus neutralization assay for seroepidemiological study.

## MATERIALS AND METHODS

### Viruses and cells

Vero cells (ATCC CCL-81) were maintained in minimum

essential medium (MEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, Biofluid, Richmond, VA, USA). Aino virus (JaNAr 28) was propagated at 37°C in Vero cells. The viral titers were determined using the Reed-Münch method (24).

### Virus neutralization test

Virus neutralization tests were performed using the method of Ishibashi et al. (2). Briefly, serum samples were first heat-inactivated at 56°C for 30 min. Next, 50 µl of serial two-fold dilutions, in duplicate rows, were mixed with equal volumes of culture medium containing 200 TCID<sub>50</sub>/100 µl of virus in flat-bottomed 96-well plates. After incubation for 1 h at 37°C, 0.1 ml of the Vero cell suspension in MEM was added to the mixture and incubated for three days at 37°C under 5% CO<sub>2</sub> in a humidity chamber. The antibody titers are expressed as the reciprocal of the highest serum dilution at which a cytopathic effect (CPE) was inhibited. A titer of 1:4 or greater was considered to be positive.

### Serum sample collection

Serum samples were obtained from the bovine serum bank maintained in the Virology Division of the NVRQS. In total, 9,921 serum samples collected between 1993 and 2001 and 23,760 serum samples between 2002 and 2007 were used (Tables 1 and 5). The serum samples collected

**Table 2.** Seroprevalence of neutralizing antibodies against Aino virus from 1993 to 2001

Area	Percent positive (%)								
	1993 (400) <sup>a</sup>	1994 (450)	1995 (390)	1996 (450)	1997 (400)	1998 (410)	1999 (2,531)	2000 (2,702)	2001 (2,188)
Kyonggi	76.0	70.0	64.0	76.0	32.0	18.0	11.1	7.7	9.0
Kangwon	50.0	32.0	24.0	14.0	26.0	6.0	3.0	6.6	2.5
Chungbuk	98.0	76.0	40.0	38.0	18.0	6.0	11.6	18.8	15.1
Chungnam	86.0	94.0	48.0	74.0	80.0	46.0	30.8	52.0	68.1
Jeonbuk	76.0	88.0	70.0	68.0	20.0	64.0	28.7	34.7	50.5
Jeonnam	89.0	46.0	NT	74.0	74.0	68.0	41.3	26.7	44.0
Gyeongbuk	NT <sup>2)</sup>	44.0	50.0	38.0	14.0	8.0	16.3	5.3	9.2
Gyeongnam	62.0	88.0	46.0	80.0	44.0	23.3	20.0	17.3	3.2
Jeju	48.0	36.0	17.5	42.0	NT <sup>b</sup>	16.7	1.7	7.2	7.3
Total	73.1	63.8	44.9	56.0	38.5	28.4	18.3	19.6	23.2

<sup>a</sup>Total number of samples tested. <sup>b</sup>NT: not tested

between 1999 and 2001 were from female cattle of a known age.

### Statistical analysis

Annual or regional seroprevalence of neutralizing antibodies against Aino virus infection were analyzed using Chi-square test supplied by FREQ procedure from SAS/STAT product (SAS Institute, 1990). Seroprevalence among different age groups was analyzed using two samples proportion test three times ( $\leq 2$  years versus 3~4 years, 3~4 years versus 5~6 years, and 5~6 years versus  $\geq 7$  years).

## RESULTS

### Annual seroprevalence of neutralizing antibodies against Aino virus from 1993 to 2001

Serum samples were collected from nine different provinces in Korea between 1993 and 2001. Approximately 400 samples were collected from 1994 to 1998, and more than 2,000 samples from 1999 to 2001. The sample numbers increased after 1999 because surveys for bovine arboviral diseases were performed regularly beginning in 1999. The average seroprevalence of Aino virus between 1993 and 2001 was 40.7% (Table 2). The highest percentage (73.1%)

was observed in 1993, and decreased thereafter. The lowest percentage (18.3%) was recorded in 1999.

### Regional seroprevalence of neutralizing antibodies against Aino virus from 1993 to 2001

Kangwon, Gyeongbuk, and Jeju showed the lowest seroprevalence percentages nearly every year ( $p < 0.05$ ). The regional seroprevalence percentages varied significantly by year. Chungnam, Jeonnam, Gyeongnam, Kyonggi, and Jeonbuk had the highest percentages from 1993 to 1998, with a little variation from year to year. Between 1998 and 2001, Chungnam, Jeonbuk, and Jeonnam had the highest percentages and maintained a consistent pattern. The average seropositive percentages in the top three areas from 1998 to 2001 were 59.3, 33.6, 37.8, and 54.2%, while the average positive percentages in the remaining six areas were between 7.7 and 13.6%.

### Seroprevalence of neutralizing antibodies against Aino virus in cows from 1999 to 2001

The serum samples collected from 1999 to 2001 were from cows over two years old, and anti-Aino virus antibody titers were determined for this group. The older cows had higher antibody titers (Table 3); cows under two years of

**Table 3.** Seroprevalence against Aino virus in cows from 1999 to 2001

Year	1999		2000		2001	
≤2 years	19.3% <sup>a</sup>	667 <sup>b</sup> (129) <sup>c</sup>	14.3%	916 (131)	15.4%	794 (122)
3~4 years	21.1%	1,435 (303)	24.0%	1,452 (348)	26.9%	1,029 (277)
5~6 years	28.9%	512 (148)	27.6%	439 (121)	37.9%	369 (140)
≥7 years	46.3%	54 (25)	46.3%	80 (37)	19.2%	52 (10)
Total	22.6%	2,668 (604)	22.1%	2,887 (638)	24.5%	2,244 (549)

<sup>a</sup> Positive rate (%) = (number positive / number tested) × 100, <sup>b</sup> Number of samples tested, <sup>c</sup> Number of positive samples

**Table 4.** Seroprevalence percentage among cattle in Jeonnam province before and after the vector season

Year	First half of the year (Before vector mosquito season, January to June)			Second half of the year (After vector mosquito season, July to December)			Incidence rates (%)
	Number of samples tested	Number of positive samples	Prevalence (%)	Number of samples tested	Number of positive samples	Prevalence (%)	
1995~1996	198	79	39.9	198	123	62.1	22.2 <sup>a</sup>
1999~2000	600	106	17.7	600	208	34.7	17.0 <sup>b</sup>
Total	798	185	23.2	798	331	41.5	18.3

<sup>a</sup> Chi-square value = 19.6, d.f. = 1, *p*-value < 0.001, <sup>b</sup> Chi-square value = 44.9, d.f. = 1, *p*-value < 0.001

age showed the lowest percentage (19.3%), while cows older than seven years of age showed the highest percentage (46.3%) in 1999. The pattern was the same in 2000. In 2001, however, the pattern changed. Cows less than two years of age showed the lowest percentage (15.4%), while cows over seven years of age showed the second lowest percentage (19.2%).

#### Analysis of seroprevalence against Aino virus in cattle before and after the vector season

Serum samples from Jeonnam area were used to examine changes in the seroprevalence percentages before and after the vector (mosquito) season. For this analysis, 396 serum samples were collected between April 1995 and March 1996, and 1,200 serum samples between June 1999 and May 2000. The seroprevalence percentages prior to the mosquito season (from January to June) were 39.9 and 17.7% in 1995 and 1999, respectively (Table 4). With the increased activity of vector mosquitoes, the seroprevalence percentages from July to December were 62.1 and 34.7%, respectively. There were apparent increases (22.2 and 17.0%) in the seroprevalence percentage after the peak of

**Table 5.** Seroprevalence of neutralizing antibodies against Aino virus from 2002 to 2007

Year	2002	2003	2004	2005	2006	2007
Sample numbers	5,280	5,280	5,280	2,640	2,640	2,640
Percent positive (%)	43.8	42.9	50.7	55.3	31.4	25.4

the vector season.

#### Seroprevalence of neutralization antibodies against Aino virus infection from 2002 to 2007

The seroprevalence of Aino virus infection was analyzed continuously after 2001. Annually, 5,280 serum samples were collected from 2002 to 2004 and 2,640 serum samples from 2005 to 2007 nationally. The lowest seroprevalence was observed in 1999 and 2000 (18.3 and 19.6%, respectively). The seroprevalence increased continuously up to 55.3% in 2005 (Table 5). The percentages then decreased to 31.3 and 25.4% in 2006 and 2007, respectively. This pattern may be due to Aino virus transmission in 2004 and 2005; however, active Aino virus isolation was not attempted during that period.

## DISCUSSION

Aino virus infection is a vector-borne bovine arboviral disease and its infection rate is affected by seroprevalence percentages. Seroepidemiological assays may be a useful tool for understanding disease status and the protective capabilities of farms. In this seroepidemiological study of serum samples collected between 1993 and 2007, it was easy to assume that the disease had existed in Korea before 1993 since vaccination against Aino virus infection had not been practiced before 2005. The seroprevalence percentage for Aino virus infection was highest in 1993 (72.0%). In 1992, there was an outbreak of Akabane disease, and it is possible that both Akabane disease and Aino virus infection were prevalent in Korea, but that only Akabane disease was diagnosed. During that time, Aino virus infection may have passed unnoticed since the clinical symptoms of the diseases are clinically indistinguishable from Akabane disease, and only Akabane disease is a notifiable disease.

According to the present study, the seroprevalence percentages of Aino virus infection have varied yearly and regionally. Seroprevalence in the Chungnam area in 2001, for example, was 86.1%, while the average seroprevalence across the nation was 25.2%. This pattern may be due to the level of vector activity in each area. To support this idea, a vector prevalence study is necessary. For example, the exact status of *Culicoides* species, which are the major Aino virus vectors in Korea, must be monitored to detect a relationship between different seroprevalence percentages and vector distribution status across regions.

Although there are many *Culicoides* species in Korea, there has not been a systematic and regular surveillance of *Culicoides* species since 1973 (25). There is a systematic and national-level arthropod vector monitoring system in Korea, which is 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 may be possible to know the exact ecology of *Culicoides* species, which would aid in the control of Aino virus infection and other bovine arboviral diseases.

The virus neutralization antibody titers were higher in the older cows. The antibody titers against Aino virus infection among cows younger than two years of age were 9.3 to 15.4%, while the titer among cows over seven years of age was 46.3%. This may be because older cows have an increased chance of coming into contact with vector mosquitoes. This assumption is the same as that made for research data collected in Kagoshima, Japan, from 1960 to 1975 (26). An analysis of serum samples collected from cows between the ages of one and twelve years in Kagoshima between 1960 and 1975 showed that antibody titers against Aino virus infection increased according to age. Cows over six years of age in Kagoshima tended to show a much higher seroprevalence percentage (80%) compared with cows in Korea (46.3%). This may be because the climate in Japan is more suitable for vector mosquitoes.

Cows over seven years of age showed a very low seroprevalence percentage in 2001. That is because most of the samples were collected from areas (Kangwon, Choongnam, and Jeju) with generally low seroprevalence percentages. Additional systematic sampling methods and continuous surveys are needed to obtain more reliable data. The collection of serum samples from farms before and after the vector season, in addition to information on vector distribution would supply reliable epidemiological data for Aino virus infection.

Overall, the seroprevalence of Aino virus infection has fluctuated twice since 1994. Its highest recorded percentages were in 1994 and 2005 (roughly a ten-year interval). It is possible that there were major bouts of Aino virus transmission between the two time points.

Presently, Aino virus infection does not pose a major threat to the bovine industry in Korea. With the advent of global warming, however, the future prospects for Aino virus transmission in the cattle industry may change. It would be beneficial to prepare a strategy for coping with climate

change-related diseases, including Aino virus infection, in advance to reduce damage. The results of this study may serve as a basis for future epidemiological studies on Aino virus infection.

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