

# Molecular and Serological Investigation of Akabane Virus Infection in Cattle in Kars-Turkey

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## ABSTRACT

Akabane virus (AKAV) is an insect-transmitted disease in ruminants that causes abortions, mummified fetuses, premature birth, stillbirth and congenital arthrogryposis hydranencephaly. This study was a serological and virological examination of the AKA virus in herds with and without abortion problems. Blood serum samples were collected for this purpose from 326 cows chosen at random; four of these cattle herds had abortion problems (n=138) and 5 cattle herds did not have abortion problems (n=188). In the same way, blood serum samples were obtained from 25 bulls in herds with abortion problems and 25 bulls in herds without abortion problems. The blood serum samples were tested with the Competitive Enzyme Linked Immunosorbent Assay (C-ELISA) for the presence of the AKAV antibody. AKAV seroprevalence rates were found to be 2.2% in cows and 2% in bulls. Serological data in cows was found to be 2.9% in herds with abortion problems and 1.6% in herds without abortion problems. In the bulls, seropositive results were only identified in one bull's blood sample from the herds with abortion problems. In the virological studies, the presence of AKAV nucleic acid was examined in 10 randomly selected seronegative and 8 seropositive blood samples using reverse transcription polymerase chain reaction (RT-PCR). AKAV nucleic acid was not found in any of the seropositive or seronegative samples.

**Keywords:** Akabane Virus; Cattle; C-ELISA; RT-PCR; Turkey.

## INTRODUCTION

Akabane is a pathological infection spread by biting midges (*Culicoides spp.*) in cattle, sheep and goats. In ruminants, the disease is observed particularly in the rainy part of the summer when the vectors are active. The agent has been isolated from midges and infected cattle in many countries (1-5). The akabane virus (AKAV) generally causes short-lived subclinical viremia without significant clinical symptoms in sensitive ruminants. The infection results in stillbirths, abortions, and congenital arthrogryposis-hydranencephaly (AH) syndrome in the 2<sup>nd</sup> and 3<sup>rd</sup> trimesters of pregnancy with lower incidence rates in the 1<sup>st</sup> trimester (5-7). However,

certain strains of AKAV are known to cause encephalomyelitis in adult cattle and calves (8-10). Significant economic losses may occur, depending on how the pathogenesis of the AKAV infection develops in pregnant animals. Akabane virus is a members of the Simbu serogroup, genus *Orthobunyavirus*, family *Bunyaviridae* (11, 12).

AKAV infection was confirmed by histopathology, immunohistochemistry, serology, and genetic analysis (13). In order to determine the specific antibodies that have developed against AKAV, methods such as serum neutralization (SN), hemagglutination inhibition (HAI) and enzyme linked immunosorbent assay (ELISA) can be used (14, 15). In addi-

tion, RT-PCR and real-time RT-PCR techniques are used to identify the viral genome of AKAV (9, 13).

The aim of this study was to define, in cattle, the molecular and serology of AKAV, which has been shown to be active in western Turkey, in three provinces in North-eastern Anatolia. In this way, we expected to obtain virological and seroepidemiological data authorizing some hypotheses on the possible AKAV circulation pathways among the neighboring regions. Another aim is to determine possible the role played by the akabane virus infection in cases of abortion in cows.

## MATERIAL AND METHODS

### Animals and Serum Samples

Blood serum samples were collected by jugular vein puncture into vacuum tubes with clot activator from randomly selected 326 cattle and 50 bull reared in private small scale production units from three provinces, i.e. Iğdır, Kars and Ardahan (Figure 1). Of the blood serum samples collected from the cattle, 138 came from four different herds in the areas of Ardahan and Iğdır, and 188 came from five different herds without an abortion problem in the Kars region (Table 1). The samples of bull blood sera came from animals in the 9 different herds of cattle used for sampling (Table 1). After clotting at room temperature for 15-30 minutes and centrifugation at 3000 g, at 4°C for 10 minutes, sera were carefully harvested, and stored at -20°C until analysis.

### Competitive Enzyme Linked Immunosorbent Assay (C-ELISA)

For detection of AKA virus antibodies a competitive ELISA system (ID VET, Product code: AKAC, France) was used.



**Figure 1:** Geographical positioning of the Turkish provinces in which the study was performed.

**Table 1:** The distribution of samples based on province and herds

Herd No.	Province	Blood serum samples from herds of cattle without an abortion problem	Blood serum samples from herds of cattle with an abortion problem	Bull blood serum samples
		Animal number	Animal number	Animal number
I	Ardahan	–	13	6
II	Ardahan	–	22	6
III	Iğdır	–	11	6
IV	Iğdır	–	92	7
V	Kars	40	–	6
VI	Kars	40	–	5
VII	Kars	40	–	5
VIII	Kars	40	–	5
IX	Kars	28	–	4
<b>Total</b>		<b>188</b>	<b>138</b>	<b>50</b>

Tests were performed according to the manufacturer's instructions. Briefly, 25 µl of test sera, and controls diluted at 1:2 in dilution buffer were added to each well. Following 90 min incubation at 37°C all wells were washed three times and anti-Akabane-HRP conjugate was added in all well as a 100 µl. Washings were performed again after 30 min incubation at 37°C. In the final step, 100 µl substrate solution tetramethylbenzidine (TMB) was added to each well and incubated for 15 min at 21°C and the reaction was stopped by adding 100 µl 0.5M H<sub>2</sub>SO<sub>4</sub>. The OD of each well was read using an ELISA reader at a wavelength of 450 nm. According to the kit procedure: the test is validated if the mean value of the negative control optical density (OD<sub>nc</sub>) is greater than 0.6 and the mean value of the positive control optical density (OD<sub>pc</sub>) is less than 50% of the OD<sub>nc</sub>.

The competition percentage (S/N) for each sample was calculated by the following formula:

$$S/N = (OD_{\text{sample}} \div OD_{\text{pc}}) \times 100$$

Samples presenting a competition percentage: greater than or equal to 40% are considered negative, between 30 and 40% are considered doubtful, less than 30% are considered positive.

### Extraction and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Ten samples randomly selected from seronegative samples and eight seropositive samples were extracted using a

High Pure Viral Nucleic Acid Kit (Roche, Mannheim, Germany), according to the manufacturer's recommendation. Amplification of cDNAs by OIAGEN OneStep RT-PCR kit (Qiagen, Hilden, Germany) was performed using the primer pairs AKAI172F/AKAI560R (5'-CAGAAGAAGGCCAAGATGGT-3'/5'-AAGTTGACATCCATTCCATC-3') for detecting the viral S RNA segment (16). Reverse transcription was conducted at 50°C for 30 min. This mixture was then heated for 95°C for 15 min to stop the reaction and to activate HotStart Taq DNA polymerase. The resulting cDNA was amplified by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 45 s followed by one step of final extension at 72°C for 10 min. Five microliters of each PCR product was analyzed in 1.5% agarose (Prona agarosa-BIOMAX, Ardoz-Madrid, Spain) gels containing ethidium bromide (Sigma-Aldrich, St. Louis, MO, USA). Akabane virus obtained from the Ankara University Faculty of Veterinary Medicine Virology Department was used as positive control.

### Statistical Analysis

Statistical analysis was carried out with Statistical Package for Social Sciences software (Chicago, IL., USA.) (17). Significant differences between male and female were evaluated using the chi-square ( $\chi^2$ ).

Furthermore, in view of the climatic differences and the herds with and without abortion problems, statistical analyses were performed using the Chi-Square ( $\chi^2$ ) test to determine whether or not there were significant differences between seropositive values in the Iğdır region and other two areas. A *P* value < 0.05 was regarded as a significant difference between groups compared.

## RESULTS

### C-ELISA

The seropositive rate for AKAV infection in the 326 cows collected as part of the study was 2.15% and for bulls 2.00%. When examined on the basis of herds, seropositivity in all of the blood sera obtained from herds with abortion problems was 2.90%, and seropositivity among these herds ranged from 0% to 4.55%. AKAV seropositivity in the blood sera obtained from herds without abortion problems was 1.6%, and seropositivity among these herds ranged from 0% to 5.0% (Table 2).

**Table 2:** The seroprevalence of AKAV infection according to the sampled herds

Herd No	Cattle samples			Bull samples		
	The number of materials	C-ELISA		The number of materials	C-ELISA	
		+	%		+	%
I*	13	0	0	6	0	0
II*	22	1	4.55	6	0	0
III*	11	0	0	6	1	16.67
IV*	92	3	3.26	7	0	0
V**	40	0	0	6	0	0
VI**	40	0	0	5	0	0
VII**	40	0	0	5	0	0
VIII**	40	2	5.00	5	0	0
IX**	28	1	3.57	4	0	0
<b>Total</b>	<b>326</b>	<b>7</b>	<b>2.15</b>	<b>50</b>	<b>1</b>	<b>2.00</b>

\* Cattle herds with abortion problems.

\*\* Cattle herds without abortion problems.

In terms of AKAV serology in bulls, on the other hand, seropositivity was not found in blood samples from herds without abortion problems, and specific antibodies formed against the AKA virus were found in the blood serum sample of only one bull from herd III, which had abortion problems.

When the regions where the sampling for the study was conducted are evaluated in terms of AKAV seropositivity (cow and bull serum combined), it was 2.13% in Ardahan, 3.45% in Iğdır and 1.41% in Kars (Table 3).

### RT-PCR

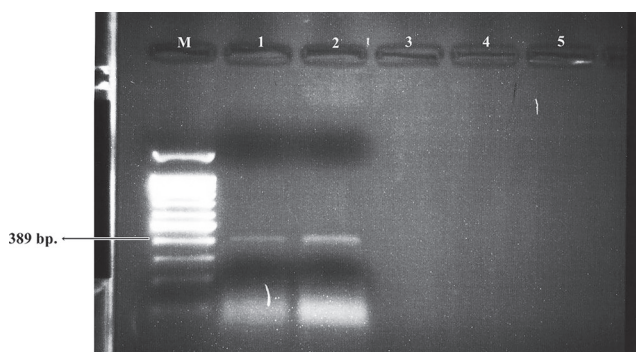
Specific PCR products of 389 base pairs were not detected in the total 18 sera samples (Figure 2).

### Statistical Analysis comparing different regions

Taking into consideration male and female cattle, herds with and without abortion problems and the climatic differences, no significant differences (*P* > 0.05) were found as a result

**Table 3:** The seropositivity rates of AKAV infection according to provinces from which the were materials obtained

Province	Animal Number (cattle+bull)	Number of positive sera	prevalence (%)
Ardahan	47	1	2.13
Iğdır	116	4	3.45
Kars	213	3	1.41
<b>Total</b>	<b>376</b>	<b>8</b>	<b>2.13</b>



**Figure 2:** The result of AKAV PCR in serum samples. Line M: 100 bp DNA Ladder; Lines 1,2: positive control (389 bp); Lines 3,4: Samples used for the study; Line 5: negative control.

of the statistical analyses of the seropositive numbers in the Iğdır region compared with the other two regions.

## DISCUSSION

To date, AKA virus has been found in Australia, China, Japan, Israel, Korea, Taiwan, and Saudi Arabia (2, 4, 5, 18-21). Its antibodies have been found in cattle from many countries in Southeast Asia, Middle East, and Africa (22-24).

The seropositive rate for AKAV infection in the 326 cows collected as part of the study was 2.2% and for bulls it was 2%. When examined on a herd basis, total seropositivity in the blood serum obtained from herds with abortion problems was 2.9% while seropositivity among the herds varied between 0% and 4.6%. AKAV seropositivity in the blood serums obtained from herds without abortion problems was 1.6%, and seropositivity among these herds ranged from 0% to 5%. In terms of AKAV serology in bulls, on the other hand, seropositivity was not found in bull blood samples from herds without abortion problems, and specific antibodies formed against the AKA virus were found in the blood serum sample of only one bull from herd III, which had abortion problems.

When the differences in seroprevalence rates between the different sample regions are examined, the reason for the high seroprevalence in the Iğdır region is thought to be due to the hotter climate and a more widespread vector population. However, the difference in seropositivity was not statistically significant ( $P > 0.05$ ). Similarly, statistical analyses found that the seroprevalence differences between males and females and between herds with and without abortion problems were insignificant ( $P > 0.05$ ).

In studies conducted in Turkey and around the world (21, 25-27), the seroprevalence of the akabane infection has been found to vary between 0.14% and 22%. The reasons for the different AKAV seroprevalence rates found in cattle both in our study and other studies maybe dependent on factors such as the number of animals sampled, local climatic conditions, breed sensitivity, age, vector distribution and sampling time.

The RT-PCR and real-time RT-PCR methods have been successful molecular tools for the confirmatory diagnosis of Akabane disease by identifying the viral genome (3, 4). They have allowed the detection of AKAV RNA in a variety of clinical samples such as blood, tissues, fetuses and swabs. In this study serum samples were used. Several PCR assays have been developed for the detection of AKAV (5, 16, 28, 29). The RT-PCR has the advantages of speed, specificity, and sensitivity for the detection of AKAV RNA. Specific probes have been developed to detect AKAV using PCR technique. The use of PCR allows sensitive confirmation of the presence of AKAV in infected animals and may also be useful for phylogenetic and epizootiological studies in both natural and AKAV susceptible hosts. We used the primer pairs AKAI172F/AKAI560R (5'-CAGAAGAAGGCCAAGATGGT-3'/5'-AAGTTGACATCCATTCCATC-3') for viral S RNA segment (16) and AKAV RNA was not detected in 18 sera samples. Even though this study found no specific DNA product (389 bp in length), this situation could be due to a low rate of infection or a lack of infectivity in serum samples tested.

In recent years, with the importation of cattle and sheep from abroad, the incidence of many diseases, especially abortion diseases, has increased in Turkey, posing serious threats to cattle and sheep industry. Surveys on abortion diseases in domestic cattle and sheep have been carried out, but most were restricted to Infectious Bovine Rhinotracheitis (IBR), Bovine Viral Diarrhea (BVD), Blue tongue (BT), brucellosis, and other diseases. In spite of akabane being one of the important pathogenic microorganisms causing abortion, akabane virus has not received adequate attention in ruminants in Turkey. In order to develop an effective prevention and control program to fight against abortion diseases in ruminants, it is important to obtain epidemiological information on the prevalence of akabane disease in cattle and sheep. To this end, we found specific antibody against akabane virus and studied the prevalence rate of the abortion disease caused by akabane virus in cattle from various North-eastern

provinces of Turkey. Even though we could not identify the viral genome in the molecular study that was conducted, demonstrating the presence of the disease serologically is an important piece of data.

Considering the Turkey's geographical location, this country forms a bridge between Asia-Europe for transition of many contagious diseases, such as the AKAV infection. Further transversal epidemiological serosurvey would be necessary for exploring the AKAV infection occurrence in this world area regrouping all borderline provinces of Turkey, Georgia, Armenia, Azerbaijan and Iran and for evidence and the preferential routes of virus circulation.

In conclusion, the results obtained in the present study showed that AKAV is not common in North-eastern Anatolia region, which is the most important cattle production area in Turkey. Consequently, sanitary programs for preventing and controlling the akabane disease may be based not only on vaccination plans and vector eradication but also on control of cattle movement between neighboring countries.

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