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Research Article

Development of an In Situ Hybridization Method for Detection of Akabane Virus

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Abstract

Akabane virus (AKAV) is an arbovirus belonging to the family Bunyaviridae, genus Orthobunyavirus. AKAV consists of three-segment (L, M, and S RNA segments), negative single-stranded RNA. The aim of this study was to investigate an *in situ* hybridization method (ISH) in a Vero E6 cell line infected with Akabane virus. The 320 base pair amplicon was obtained by RT-PCR with a primer pair and labeled with digoxigenin. Akabane virus RNAs were seen as a granular pattern in the cytoplasm of infected cells.
As a result, the expression of the particular Akabane virus gene area was successfully disclosed in the current investigation using the ISH method with a digoxigenin-labeled probe.

Keywords: Akabane virus, in situ hybridization, method, pathology

1 INTRODUCTION

Akabane virus (AKAV), transmitted by hematophagous arthropods such as Culicoides, is an arbovirus belonging to the Bunyaviridae family in the genus Orthobunyavirus. In 1959, the prototype AKAV strain was first isolated from mosquitoes in the central region of Japan [1,2]. AKAV consists of three-segment (L, M, and S segments), negative single-stranded RNA. The L protein, which has RNA polymerase activity for viral genome replication and transcription, is encoded by the L RNA segment. The M segment encodes two viral envelope glycoproteins necessary for viral neutralization and the non-structural protein NSm, which is formed from a precursor polypeptide by post-translational cleavage. The nucleocapsid (N) protein and a smaller non-structural (NSs) protein are both encoded by the S-RNA segment in overlapping reading frames [3]. Premature births, stillbirths, and congenital abnormalities (arthrogryposis-hydranencephaly syndrome) in cattle, sheep, and goats are caused by AKAV in both epizootic and sporadic outbreaks [4,5,6]. Calves and adult cattle have been known to develop encephalomyelitis caused by an Akabane virus type called Iriki [7,8]. The epidemics have regularly resulted in significant financial losses to the livestock industry and have been observed in Japan, Korea, Taiwan, Australia, Israel, and Turkey [6,9,10].

In our previous study, we isolated the virus and immunohistochemically and molecularly demonstrated Akabane virus (AKAV) infection in a 15-day-old lamb in Aydin province, Turkey, which suffered from ataxia, incoordination, stagnation, diarrhea, and paralysis. This paper describes the first molecular characterization of Turkish Akabane virus strains in small ruminant flocks based on partial sequence analysis and pathological findings. Sequence analysis based on the incomplete S segment of the isolates revealed that the virus is close to the II genogroup in the phylogenetic tree. Immunohistochemistry showed that the AKAV antigen

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© 2024 Journal of Biological Methods published by POL Scientific

Received 2023-11-03; Revision received 2023-12-20; Accepted 2023-12-25; Published 2024-02-23

Access this article online

Quick Response code:

Website: http://jbm.polscientific.com

DOI: 10.14440/jbm.2024.413

How to cite this article: Toplu N, Oğuzoğlu TC, Akkoç AN. Development of an In Situ Hybridization Method for Detection of Akabane Virus. *J Biol Methods* 2024;11:e99010011. DOI: 10.14440/jbm.2024.413

was mainly localized in the neurons of the brainstem and spinal cord of lamb and calf, which provided crucial information not only for diagnosis but also for the understanding of mechanism of the disease [11].

Currently, immunohistochemistry is used in pathology laboratories to diagnose viral infections by detecting viral antigens with polyclonal or monoclonal antibodies. However, in order to detect viral antigens, viral proteins must be produced, and their antigenicity must be obtained using tissue preparation techniques. According to Toplu and Alçığır [12] and Toplu *et al* [13], the sensitivity of viral antigen detection by immunohistochemistry in brain sections from chickens infected with avian encephalomyelitis virus may be significantly reduced as a result of circulating antibodies, impaired viral protein synthesis, and/or rapid tissue destruction, including inflammation and necrosis. In situ hybridization has been used for three decades in pathology laboratories as a more sensitive molecular method for detecting viral nucleic acids. This technique has the great advantage of allowing for the detection of virus-specific DNA or RNA sequences in tissues/cells compatible with microscopic examination. ISH is an important molecular biology method widely used in pathology laboratories because it is based on the determination of DNA or RNA sequences similar to PCR and represents the histological morphology of tissues as in immunohistochemistry [14]. This method has been successfully used in the diagnosis of arthropod-borne viral infections such as West Nile virus, bluetongue and border disease virus or in the elucidation of disease mechanisms [15,16]. In the present study, the ISH method, which had only been used in one experimental study using digoxigenin-labeled probes, was performed on Vero cell lines (Vero E6) infected with Akabane virus to determine AKAV nucleotides.

2 MATERIALS AND METHOD

2.1 Cell line and virus inoculation

Vero E 6 line was gradually thawed on ice block from -80ºC laboratory stock, then gently centrifuged in order to separate from DMSO and was ready for monolayer cultivation. For cultivation, Dulbecco's Minimum Essential Medium (DMEM) including 5% fetal calf serum (FCS) was used. 12-well plate was prepared for virus inoculation and ISH. Akabane virus, previously isolated by Oğuzoğlu *et al* [11] in our department, was inoculated to cell line. Fixation with 2% paraformaldehyde solution for 10 minutes were started zero (0) hour post inoculation (hpi). We continued the fixation process at 12, 24, 48, 72 hpi and followed up the cytopathogenic effects per each inoculation time period.

2.2 PCR Amplification and Digoxigenin Labelling

RNA isolation and amplification were performed on stock virus inoculum in which we had previously inoculated Akabane virus into Vero E6 cell lines. Supernatant was separated and genome extraction was performed by using commercial viral RNA/DNA isolation kit (PureLink, Invitrogen). After cDNA was generated by using RevertAid First Strand cDNA Synthesis kit (Thermo Scientific), nested PCR was accomplished by previously reported primers for Akabane S segment detection [17]. Both amplicons, obtained in the first and second round respectively, were run in 1% agarose gel and visualized under UV transilluminator.

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 PCR 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.

In order to perform In Situ Hybridization, relevant probe was generated based on obtained purified amplicon (230 bp) by commercial digoxigenin (DIG)-labelling kit (DIG Probe Synthesis Kit No. 11636090910, Roche, Mannheim, Germany). After labeling, the probe was run in 1% agarose gel and then the size was confirmed by visualization under a UV transilluminator (**Fig. 1**).

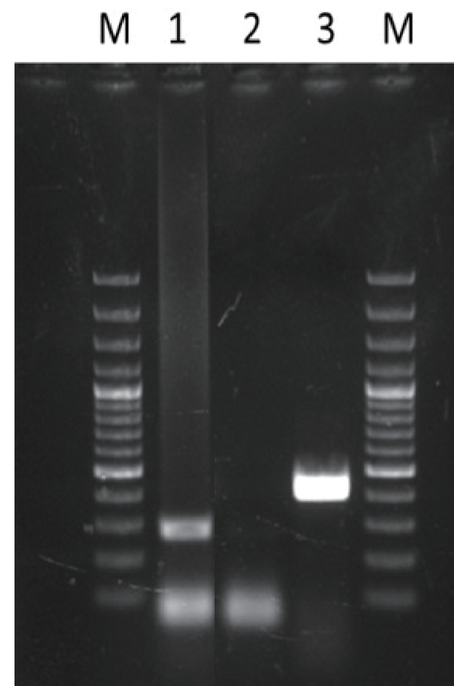

Figure 1. M= 100 bp marker, 1= nested PCR positive amplicon, 2= negative control 3= Digoxigenin labeled prob from nested PCR product (320bp)

2.3 In situ Hybridization

ISH was performed on each cell culture well with the DIG-labeled probes and the in-situ hybridization kit (K2191050, Biochain, Eureka Drive Newark, CA). The optimal conditions, including probe dilutions and hybridization temperatures, were determined by titration experiments. Cells were rehydrated through a graded series of ethanol, and then washed for 5 minutes in sterile

deionized water containing 0.2% RNase block. Cells were fixed in 2% paraformaldehyde in DEPC-PBS at room temperature for 10 minutes. After washing twice with DEPC-PBS at room temperature for 5 minutes, cells were treated with 10 µg/ml Proteinase K (Roche) at 37 ºC for 10 minutes. Cells were washed with DEPC-PBS for 5 minutes before re-fixation in 2% paraformaldehyde in DEPC-PBS and then washed with DEPC-water. They were treated with pre-hybridization solution at 50 ºC for 3-4 hours. Hybridization was carried out overnight in a humidity chamber at 42 ºC in a mixture containing heat-denatured DIG-labeled probes (2-4 ng/µl) in hybridization solution. Post-hybridization washes comprised washing in 2xSSC for 10 minutes at 45 ºC, 1.5xSSC for 10 minutes at 45 ºC and twice with 0.2xSSC for 20 minutes at 37 ºC, before blocking with 1% blocking solution. Then cells were incubated with AP-conjugated anti-digoxigenin antibody (1:500 PBS diluted) for overnight at 4 ºC. Subsequently, cells were washed with PBS three times at room temperature, for 10 minutes each time. After washing with PBS, cells were treated with 1xAlkaline Phosphatase buffer twice at room temperature, for 5 minutes each time. Finally, cells were incubated for 1 hour in the dark with NBT-BCIP substrate. Following this procedure, the wells were washed, and then sealed with a water-based sealer. ISH staining within the cells was observed and captured on camera using an inverted microscope.

The scoring of ISH labeling was based on the proportion of positively stained cells observed in a total of 10 high-power fields measured at 400X. The scores were as follows: - : absent; (+): weak immunopositivity (1-5% positive cells); (++): moderate immunopositivity (6-10 % positive cells); (+++): strong immunopositivity (>11% positive cells).

3 RESULTS AND DISCUSSION

AKAV RNAs labeling was observed as granular patterns in the cytoplasm of dispersed cells infected with AKAV after six hours of incubation of infected cell lines (**Fig. 2**). During the first 12, 24, 36, and 72 hours of incubation, cells primarily contained viral RNAs (**Table 1**). No positive reaction was observed in control wells that included virus-free cell lines. Additionally, ISH using BVDV probe yielded negative results with positive control cells.

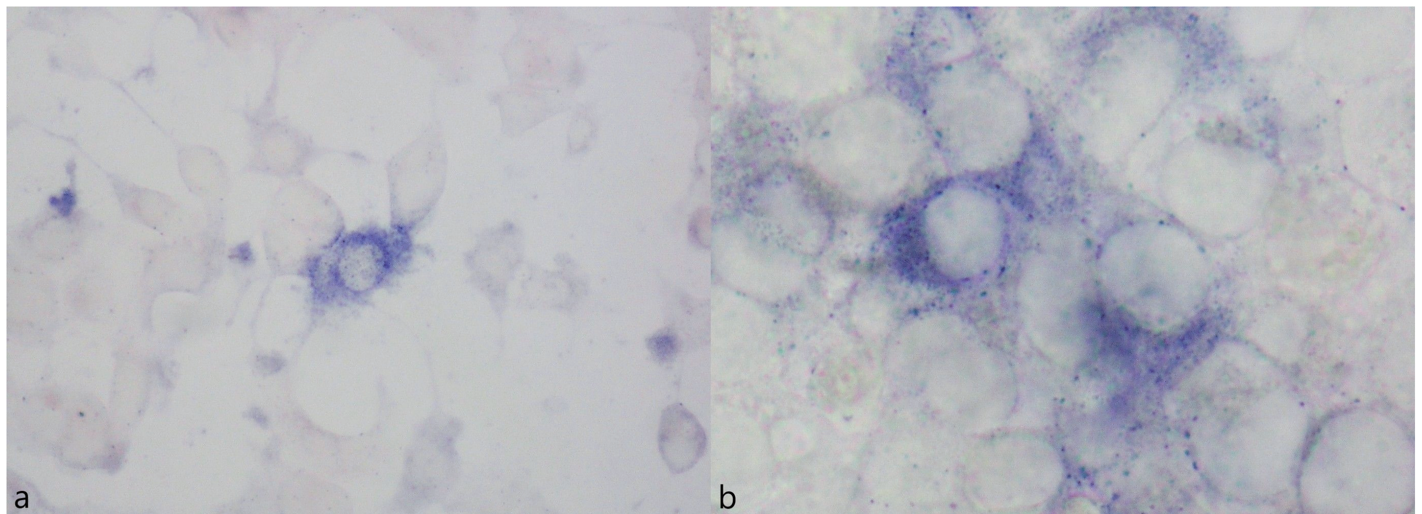

Figure 2. ISH AKAV RNA labeling in cell cultures. a. A few positive cells at 12 hpi (10x) **b.** Large number of cells at 72 hpi (40x).

Table 1. The scores of ISH labeling based on the proportion of positively stained cells.

Control	12 hpi	24 hpi	48 hpi	72 hpi
-	+	++	+++	+++

In situ hybridization is a technique based on the detection and localization of specific RNA or DNA molecules in cells, tissue sections, or whole tissues [18]. The method, which has been used in molecular and cytological pathology since the 1970s, became a significant technique in cellular pathology in the 1980s [19,20]. Due to the use of radioisotope-labeled probes that could be hazardous to human health, the application and routine use of ISH were more limited than immunohistochemistry during these years. With the advent of FITC-, digoxigenin-, and biotin-labeled probes, the use of ISH has become easier. The ISH technique works by forming a hybrid molecule between the endogenous single-stranded RNA or DNA in the cell and a complementary single-stranded RNA or DNA probe. After hybridization with the compatible sequences, the labeled probe is visualized with fluorescent or non-fluorescent chromogens [18]. Nucleic acid probes are as important for ISH as primary antibodies are for

immunohistochemistry and are referred to as labeled nucleic acids [20]. The specificity of the probes used is critical for increasing the accuracy and sensitivity of the method [21]. However, the correct applicability of ISH requires consideration of factors such as proper sampling, processing of samples, possibility of false positive results in cross hybridization, early-stage infection, latent infection (low copy number per cell), and the need for assistance from other methods in some cases [22]. In the current study, the probe was generated by PCR amplification of a 320 base pair gene sequence region unique to the S segment of AKAV. On the other hand, the probe was labeled with digoxigenin, which we had previously used successfully to identify West Nile virus and Border disease.

Since ISH provides rapid and accurate results, which can be analyzed and interpreted together with histological findings for diagnosing microorganism infection and revealing disease mechanisms, it is a more suitable technology than liquid phase PCR. To better understand the pathophysiology of the disease, viral load can be determined in cells and tissue samples by using ISH. The method has the advantage of revealing the target cells specific to each virus and tracking the progression of the disease [23]. The visualization of AKAV virus replication sites and viral load in tissues using the ISH approach in conjunction with tissue morphology has contributed significantly to the understanding of the pathophysiological mechanisms of the disease in the experimental study by Kimura *et al* [24]. Similarly, in the current study, ISH was used to successfully detect virus replication and cellular localization in Vero cell lines using probes that determine the S segment.

The sensitivity of the ISH method is dictated by the type of probe used, especially when the viral nucleic acid copy number is low. This condition is often difficult, especially in formalin-fixed and paraffin-embedded tissues [23]. However, the cell culture inoculation method used in our current study eliminated this drawback and demonstrated the specificity of the probe and, thus, the method.

ACKNOWLEDGMENT AND/OR DISCLAIMERS FUNDING

No specific funds or donations were received for this study.

DECLARATION OF COMPETING INTERESTS

The authors declared no conflicts of interest in relation to the research, authorship or publication of this article.

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