Detection and Molecular Epidemiology of Bovine Kobuviruses in Calves With Acute Gastroenteritis for the First Time in Iran

Ahmad Nazaktabar*1,2

1Department of Pathobiology, Faculty of Veterinary Medicine, Amol University of Special Modern Technologies, Mazandaran, Iran

Abstract
Background: Calf diarrhea is an important issue in cattle farms. Although rotavirus A is the primary viral agent causing calf diarrhea, infectious causatives of diarrhea remain unknown in many cases. Bovine kobuviruses are almost newly detected enteric viruses that have not been studied extensively. There is no information about the epidemiology and prevalence of kobuvirus and its importance in calf diarrhea in Iran.

Objectives: The molecular epidemiology and phylogeny of kobuviruses were investigated in one-month-old diarrheic calves, and rotavirus A was simultaneously surveyed to find the outbreak rate of the co-infection of both viruses in diarrhea.

Materials and Methods: This study investigated 200 fecal diarrheic samples obtained from one-month-old calves using reverse transcription polymerase chain reaction (RT-PCR) methods. Samples were collected from rural and industrial cattle farms located in 7 provinces of Iran. The 3D domain of the RNA-dependent RNA polymerase (RdRp) enzyme of three positive samples from Mazandaran, Fars, and Isfahan provinces was subjected to the phylogenetic study.

Results: It was found that 27 specimens are positive for kobuvirus. Although the frequency of rotavirus A detection was 24% (48 out of 200), co-infection was observed in 5 samples. Moreover, phylogenetic analysis showed a low relationship between the sequenced samples, indicating that the circulating bovine kobuviruses originated from different ancestors.

Conclusion: The results showed that bovine kobuvirus with different phylogenetic origins is highly prevalent in cattle farms in Iran. Regarding the low rate of co-infection with rotavirus A, bovine kobuviruses should be considered an important enteric viral agent in calf diarrhea.

Keywords: Calf diarrhea, Kobuvirus, Molecular epidemiology, Phylogenetics, Rotavirus A

Background
Diarrhea in calves is one of the most common problems of dairy farms. In addition to incurring various economic losses, this disease can be responsible for more than 50% of neonate calf deaths.1 Bovine rotavirus A (BRV-A), bovine coronavirus, and bovine viral diarrhea are the three main viruses causing diarrhea. Although routine tests are available for these three viruses, they are not detected in all clinical cases of diarrhea, and many specimens remain undiagnosed.2,3 On the other hand, there is scant information about the epidemiology of other enteric viruses such as bovine kobuviruses (BKV), astroviruses, toroviruses, and calciviruses as well as their role in calf diarrhea. Most of these viruses are probably not a primary pathogen and, along with other predisposing factors, can cause and/or exacerbate diarrhea syndrome in calves.4

BKV belongs to the Aichivirus B, one of the 6 official species (Aichivirus A to F) classified as in the genus Kobuvirus and the family Picornaviridae. A cubic capsid without an envelope surrounds the BKV's single-stranded viral RNA genome (8.3 to 8.8 kb).4,5 Following the first detection of BKVs as a contaminant agent of cell cultures in Japan, it has been reported in the feces of dairy and feedlot bovine calves and adults in numerous countries; however, its pathogenicity remains controversial. Recent studies in the United States, Canada, and Egypt have suggested that the virus may be a primary infectious agent that causes diarrhea.5,6

There is no information about the epidemiology and phylogeny characteristics of BKV in Iran. Hence, the present study investigated BKV in 7 provinces of Iran for the first time to determine its prevalence in calf diarrhea.

Materials and Methods
Two hundred fecal samples gathered from calves aged under one month with symptoms of diarrhea were collected from industrial and rural farms in Tehran, Alborz, Qazvin, Mazandaran, Khorasan Razavi, Sistan and Baluchestan, Fars, and Isfahan for more than six years from autumn 2010 to winter 2017. The presence of BKVs was monitored by primer pairs UNIV-kobu-F (5’-GTGTTTTRATGATGTTGTTGA-3’)

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and UNIV-kobu-R (5'-TGGAYTACAAG(R) ATGTTTGTGTC-3') which are specific to the 3D domain of the BKV RNA-dependent RNA-polymerase (RdRp) enzyme.² BRV-A was surveyed using specific primer pairs to detect the VP6 gene according to the manner described in previous studies.²,⁹

The extraction of the viral genome was performed using the Viral Gene-spin™ Extraction Kit (Intron company, Korea) according to the manufacturer's instructions by the explanation that 300 microliters of 20% suspension of stool samples in phosphate-buffered saline (v/v) were added to 700 µL of the lysis buffer. Following the extraction, two microliters of the extracted RNA were applied in a reverse transcription polymerase chain reaction (RT-PCR) using the one-step RT-PCR Qiagen kit according to the method previously described.²,⁹,¹⁰ Three positive samples from Mazandaran, Fars, and Isfahan provinces were randomly selected for direct sequencing (Bioneer Co. Korea). Phylogenetic analysis was then performed by MEGA11 software.¹¹ The phylogenetic tree was constructed via the maximum likelihood method: Kimura's two-parameter model (gamma distributed with invariant sites) and 500 bootstraps replicate.¹²

**Results**

BKV was detected in 27 stool samples (13.5%), and rotavirus A was seen in 48 samples (24%) from which 5 samples were co-infected with BKV (2.5%). The sequenced PCR products from Isfahan, Fars, and Mazandaran provinces were deposited in the GenBank database under accession numbers MH424413, MH424414, and MH424415, respectively (Table 1).

Nucleotide identity between sequences from Isfahan and Fars provinces was 87.5%, and their identity to Mazandaran province was 91.57% and 91.95%, respectively. Sequences obtained in this study also showed 88-89% nucleotide identity to the reference strain U-1.

In the phylogenetic analysis, the sequence from Mazandaran province (accession No. MH424415) was clustered with Japanese (K-3, K-4, and K-6) and some Chinese (13/LN, 10/LN, and C1/SD) strains. However, the sequence from Fars province (accession No. MH424414) was located beside a sequence from Turkey (BOLAT113-16-TUR), and the sequence from Isfahan province (accession No. MH424413) was clustered with strains from Scotland (SC851 and SC848), as depicted in Figure 1.

**Discussion**

Diarrhea is one of the primary causes of calf death within the first weeks of their life.¹ Escherichia coli, Cryptosporidium parvum, BRV-A, and Bovine coronavirus are the most frequent infectious pathogens causing diarrhea in dairy and beef calves.²,³ However, these agents cannot be detected by the standard methods in many clinical samples; therefore, many clinical cases remain unknown.³ Kobuviruses were first detected in Japan as a contaminant agent of cell cultures fed with bovine calf serum instead of fetal bovine serum in 2003.¹⁰ Nevertheless, there is evidence that it has been rarely considered as an agent of calf diarrhea; hence, few investigations have been conducted regarding its epidemiology and pathogenic importance. Nevertheless, it has been detected in the fecal samples of both healthy and diarrheic calves with high prevalence rates in many countries worldwide.³,¹³

The pathogenicity of BKVs is still under question. However, there is some evidence suggesting that BKVs may be able to cause gastroenteritis in calves. In one study, BKV was the only virus detected in the feces of a necropsied calf with diarrhea that histopathologically indicated evidence of viral inflammation in the intestine.⁶ Furthermore, in some epidemiologic surveys, the prevalence of BKV in diarrheic calves was higher than in healthy ones. For instance, in one study in China, the frequency of BKV detected in the calves suffering from diarrhea was 35.42%, significantly higher than that in healthy calves (11.69%).¹⁴ Although the prevalence of BKV in the bovine population is noticeable and should be considered an essential enteropathogen in calf diarrhea, there is no evidence to cause a notifiable disease in humans.¹⁵

The present study investigated the prevalence of BKVs compared to BRV-A in diarrheic one-month-old calves. BRV-A was detected in 24% of the samples, of which 5 specimens were co-infected with BKV. The prevalence of BRV-A infection was in agreement with the last results of other research in which molecular methods (RT-PCR technique) were used. In these studies, the prevalence of BRV was estimated between 20-30% in Iran.⁵,¹⁵-¹⁷ The low rate of coinfection of rotavirus A and kobuvirus was noticeable, indicating that the high rate of kobuvirus infection occurs independently of BRV in Iran's farms. However, more microbiological and pathological analyses are needed to detect the co-infection of other enteric pathogens to determine in what percentage of these samples, kobuviruses as a primary pathogen

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**Table 1. The Prevalence of BKV and BRV-A in Each Province**

<table>
<thead>
<tr>
<th>Provinces</th>
<th>No. of Samples</th>
<th>BKV (%)</th>
<th>BRV-A (%)</th>
<th>Co-infection of BRV-A and BKV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alborz</td>
<td>15</td>
<td>2</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Fars</td>
<td>30</td>
<td>4</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Isfahan</td>
<td>30</td>
<td>6</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Mazandaran</td>
<td>36</td>
<td>14</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Razavi-Khorasan</td>
<td>34</td>
<td>2</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Sistan and Baluchistan</td>
<td>25</td>
<td>8</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Tehran</td>
<td>200</td>
<td>48</td>
<td>27</td>
<td>5</td>
</tr>
</tbody>
</table>

**Note.** BKV: Bovine kobuvirus; BRV-A: Bovine rotavirus.
caused diarrhea.

The distribution of BKS infection in the studied provinces was not equal. Mazandaran was the most contaminated province, whereas BKV was not detected in any specimens in the Sistan and Baluchestan province located in southeastern Iran. The climate of Mazandaran is temperate and humid, and its annual rainfall is much higher than that in other parts of Iran. The average annual precipitation in the Caspian coastal plain in Mazandaran province is about 500 mm, and many cities and villages with high human and livestock population density are located in Mazandaran province. These factors provide a favorable environment for viruses to survive and find new hosts. In addition, it should be noted that samples of this province were collected from high-density industrial farms. In contrast, the human population and livestock density are extremely scant in the Sistan and Baluchestan province. Moreover, most of the farms of the Sistan and Baluchestan province are not industrial, so all the samples were collected from low-density rural backyard farms. On the other hand, the climate of Sistan and Baluchestan province is hot and dry, which provides a desiccating condition and reduces viral survival. Since this study examined several provinces with different climates and geographic conditions, it can be concluded that the obtained result displays good insight into the epidemiology of BKV in Iran and even in the Middle East.

More than 360 nucleotide sequences of BKVs have been deposited in GenBank to date, of which only 6 are complete sequences, and most of the partial sequences in GenBank belong to the 3D segment of the viral RdRp gene (211 sequences). RdRp gene is an appropriate target for detecting these viruses in epidemiologic studies. Therefore, it has been more sequenced than the VP1 gene and is still a tool for phylogenetic analysis of BKSs. In the present study, phylogenetic analysis and nucleotide identity of the partially sequenced 3D segment of three samples indicated that BKVs circulation in Iran is highly divergent. One of these sequences (from Fars province) showed a high degree of resemblance to a strain from Turkey (one of the neighboring countries to Iran), meaning that both have common ancestors. On the other hand, the sequence obtained from Isfahan province was clustered with sequences from Europe (Scotland), and another nucleotide sequence obtained from Mazandaran province was related to Asian BKVs (Japanese and Chinese). Since the RdRp gene was conserved, and the nucleotide identity between the sequences of this study was 87.5%-91.95%, it appears that they have originated from different ancestors and different geographical areas.

**Conclusion**

In the present study, results of the epidemiology study of BKSs indicated that it is extremely prevalent in the stool...
samples of diarrheic calves. To the best of our knowledge, it is the first report on molecular epidemiology and detection of BKSs in Iran; accordingly, the obtained data can contribute to further understanding of the epidemiology of BKS as a new candidate for causatives of calf diarrhea. According to the result of the phylogenetic analysis of this study, it is suggested that complete sequencing or at least nucleotide sequences of the VP1 gene should be determined and considered for further analysis.

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Competing Interests
The author has no relevant financial or non-financial interests to disclose.

Ethical Approval
No ethical issue.

References