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Serological Investigation of *Bovine Enterovirus* in Calves in Konya Province*,**

H.Sercan PALANCI[1,a], Oğuzhan AVCI[1,b], Irmak DİK[1,c], H.Pelin ASLIM[1,d], Rüveyde GÜLBAHÇE[1,e], Oya BULUT[1,f]

[1]Selcuk University, Faculty of Veterinary Medicine, Department of Virology, Konya-TÜRKIYE
ORCID: [a]0000-0001-5408-9176; [b]0000-0001-9299-4695;[c]0000-0003-2516-9489;[d]0000-0001-9160-1255; [e]0000-0002-3665-4642; [f]0000-0002-2407-7390

Corresponding author: Rüveyde GÜLBAHÇE; E-mail: gulbahceruveyde@gmail.com
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Abstract: *Bovine enterovirus (BEV)* infection is a common viral disease in cattle. Although the infection is often subclinical, it is among the possible causes of gastroenteritis. *BEV* infections have also been associated with respiratory and reproductive system disorders and signs of diarrhea. *BEV* is divided into two serotypes, *enterovirus serotype E (EV-E)* and *enterovirus serotype F* (EV-F). This study aims to determine the seroprevalence of *EV-E* in calves in Konya province. For this purpose, 504 *BEV* unvaccinated calf blood serum samples from the Selcuk University Veterinary Faculty Virology Department laboratory were used. A serum neutralization test (SNT) was used to determine *EV-E* seroprevalence. 342 (67.85%) samples were found to be seropositive, and 162 (32.14%) were seronegative. In addition, as the serum neutralization 50 (SN_{50}) antibody titers of seropositive animals were examined, the titers determined as 1/10, 1/15 and 1/20. Among the results we obtained, the highest antibody titers were defined as 1/80 and 1/120; the total number of animals with these values was In conclusion, *bovine enteroviruses* are an infection of importance for cattle breeding. Therefore, necessary precautions must be taken to protection infection. It is thought that the results of the present study will provide important data for future studies.
Keywords: Antibody, bovine enterovirus, serum neutralization test

Konya Bölgesi'ndeki Buzağılarda *Bovine Enterovirus*'un Serolojik Araştırılması

Öz: *Bovine enterovirus (BEV)* sığır popülasyonunda yaygın olarak gözlenen viral bir enfeksiyondur. Enfeksiyon daha sıklıkla subklinik olmasına rağmen, gastroenterit hastalığının olası nedenleri arasında yer almaktadır. BEV enfeksiyonları aynı zamanda solunum ve üreme sistemi bozuklukları ve ishal bulguları ile de ilişkilendirilmektedir. *BEV, enterovirus serotip E (EV-E)* ve *enterovirus serotip F (EV-F)* olmak üzere iki serotipe ayrılır. Bu çalışma Konya bölgesinde buzağılarda *EV-E* seroprevalansını tespit etmek amacı ile yapıldı. Bu amaçla Selçuk Üniversitesi Veteriner Fakültesi Viroloji Anabilim Dalı laboratuvarında bulunan 504 adet *BEV* yönünden aşısız buzağı kan serum numuneleri kullanıldı. Kan serum örneklerine *EV-E* seroprevelansının belirlenmesi için serum nötralizasyon testi (SNT) uygulandı. 342 (% 67.85) örnek seropozitif 162 (%32.14) örnek ise seronegatif tespit edildi. Ayrıca seropozitif hayvanların Serum Nötralizasyon 50 (SN_{50}) titreleri incelendiğinde nötralize edici antikor titreleri 1/10, 1/15 ve 1/20 olarak tespit edildi. Sonuçlar arasında en yüksek antikor titreleri1/80 ve 1/120 olarak tanımlandı ve bu değerlere sahip total hayvan sayısı ise 18 olarak tespit edildi. Sonuç olarak, sığır enterovirusları sığır yetiştiriciliği için önem arz eden bir enfeksiyondur. Bu nedenle enfeksiyondan korunmak için gerekli önlemlerin alınması gerekmektedir. Mevcut çalışmadan elde edilen sonuçların gelecekte yapılması planlanan çalışmalar için önemli veriler sağlayacağı düşünülmektedir.
Anahtar kelimeler: Antikor, serum nötralizasyon testi, sığır enterovirus

Introduction

Bovine enterovirus (BEV) is an *Enterovirus* in the *Picornaviridae* family. *Picornaviruses* are small, non-membrane, positive-stranded RNA viruses (Candido et al., 2019). The *Picornaviridae* family consists of 63 genera and 147 species. Members of this family include *Aphtovirus (foot-and-mouth disease virus, equine rhinitis A virus), Cardiovirus (cardiovirus A and B), Hepatovirus (hepatovirus A), Enterovirus (A, B, C, D, E, F; Rhinovirus A, B, C), Parechovirus (A and B), Kobuvirus (aichivirus A) and Senacavirus (senacavirus A)* (Zell et al., 2017). Members of *Enterovirus* species *A* to *D* and *Rhinovirus* species *A* to *C* cause infections primarily in humans, *E* and *F* in cattle, *G* in pigs, and *H* and *L* in monkeys (Rao, 2021).

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BEV is usually isolated from healthy animals. Despite *BEV* infects mammals orally, this pathogen is unaffected by the gastrointestinal tract, travels from the digestive tract to the lymph nodes, and elicits an antibody response. Neutralizing antibodies specific to *BEV* have been detected in many species, including humans, cattle, sheep, goats, dogs, horses, and monkeys (Luo et al., 2023). Although the disease usually presents as a subclinical infection with mild symptoms, fatal cases with low morbidity have rarely been reported (Blas-Machado et al., 2007). Furthermore, the pathogenesis and virulence of the infection are still not fully understood and are currently under investigation (Gür et al., 2008).

Serotyping of *BEV* has been performed by classification studies based on tests such as hemagglutination, virus neutralization, and complement fixation. The *Enterovirus* genus consists of subfamilies with more than 80 serotypes that can infect cattle and other animal species. *BEV* is divided into two serotypes, *Bovine enterovirus serotype 1 (BEV-1)* and *Bovine enterovirus serotype 2 (BEV-2).* According to the 9th International Committee on Taxonomy of Viruses (ICTV) report, *bovine enteroviruses 1* and *2* have recently been named *Enterovirus E (EV-E)* and *Enterovirus F (EV-F)* types. In *BEV* seroprevalence studies conducted in Turkey, *EV-E* serotype was mostly detected (Gür et al., 2006; 2008; 2019; Erol et al., 2020). The host of *EV-F* was determined as domestic cattle, unlike *EV-E* (Gür et al., 2019).

EV-E has been reported from domestic cattle *(Bos taurus),* buffalo *(Bubalus bubalis)* (Urakawa and Shingu,1987), sheep *(Ovis aries)* (Jain and Batra, 1985), goats *(Capra hircus)* (Jain and Batra,1985), sika deer *(Cervus nippon)* (Urakawa and Shingu,1987), wild African buffalo *(Syncerus caffer),* and impala *(Aepyceros melampus)* (Hamblin et al., 1985). In the last two decades, the *EV-E* serotype has been isolated from a pregnant 2-year-old heifer in Oklahoma (Blas-Machado et al., 2007). Antibodies to *EV serotype E* can also be detected in species such as buffalo *(Bubalus bubalis)* (Gür et al., 2006), dolphins (Nollens et al., 2009), alpacas (Mc Clenahan et al., 2013), and rats (Zheng, 2007). The susceptibility of young calves to *BEV* infection is important for understanding the pathology associated with infection and for the epidemiology and characterization of *EV-E* infection in herds (Blas-Machado et al., 2007). *BEV* isolates were first named enteric cytopathogenic bovine viruses (Castells and Colina, 2021), and these isolates have been described in many countries worldwide (Beato et al., 2018; Ren et al., 2020; Mosena et al., 2022). BEVs found in cattle worldwide generally cause asymptomatic infections and are shed in the feces of infected animals (Park et al., 2009). Most enteroviruses are transmitted in animals and humans via the fecal-oral route and colonize the digestive tract (Rao, 2021).

Clinically diseased cattle may have extensive replication in many organs in addition to local lymph nodes, followed by reproductive, respiratory, and enteric symptoms (Solomon et al., 2010). The most common clinical signs in *BEV*-infected cattle are abortions, stillbirths, infertility, and neonatal death (Christianson, 1992; Beato et al., 2018). Persistent diarrhea, respiratory disease, and enteritis are common pathological signs in cattle infected with *BEV*. Moreover, they do not have characteristic pathological lesions distinguishing *BEV* infection from other pathogens that cause similar clinical signs (Candido et al., 2019). It is highly resistant to environmental conditions (Birdane and Gür, 2019). No vaccine prevents or controls *BEV* infection (Wang et al., 2012).

Infected cattle can easily spread *BEV* through their feces. Wild ruminants are thought to play a role in transmitting *BEV* to domestic cattle (Gras et al., 2017). In general, there are few studies on *BEV* in Türkiye. This study was conducted to determine the seroprevalence of *EV-E* infection cases in the field by detecting the presence of *EV-E* antibodies in calf populations in the Konya province and measuring antibody levels of positive samples.

Materials and Methods

Collection of samples

In this study, 504 calf blood serum samples taken from Konya province, available in the laboratory of Selcuk University Faculty of Veterinary Medicine Department of Virology, were used. All procedures were approved by the Selcuk University Veterinary Faculty Ethics Committee (Ethical approval number 2021/117 on 20/10/2021).

Confirmation of EV-E isolates by RT-PCR method

The presence of *BEV* in the virus suspension used for the study was checked and confirmed using primer sets designed for the viral polyprotein gene segment. These primer sets (*BEV*-F: 5'ACC TTT GTA CGC CTG TTT TCC-3′; *BEV*-R: 5'-GAT TAG CAG CAT TCA CGG C-3') were designed by Blas-Machado et al. (2011) to detect the presence of the *EV-E* Oklahoma isolate.

RNA extractions were performed using a commercial extraction kit (QIAamp Viral RNA Mini KitCatno: 52904) from the viral suspension produced in the susceptible cell line. The application was performed according to the protocol indicated by the manufacturer. The obtained extraction products were subjected to a one-step reverse transcriptase-polymerase chain reaction (one-step RT-PCR). The MegaFi™ One-Step RT-PCR (Cat. No.: G597) kit was used in this method. PCR cycles of the prepared reaction mixtures: after cDNA generation at 60°C for 15 min, denaturation at 98°C for 10 sec, annealing at 54°C

for 20 sec, extension at 72°C for 40 sec during one cycle of 30 sec at 98°C and 33 reactions. Finally, the last extension step was performed at 72°C for 2 min, and the RT-PCR process was terminated. At the end of the process, the PCR products were transferred to wells of a 2% agarose gel containing ethidium bromide. The products were subjected to electrophoresis. At the end of the procedure, the PCR product was visualized under UV light, and the sample was found to be positive for *EV-E* with a band in the range of 484 bp.

Cell line and virus strain

The cell lines and virus strains were obtained from the cell and virus collections of Selcuk University, Faculty of Veterinary Medicine, Department of Virology. Before starting the study, the fetal bovine serum (FBS) and the cell line to be used in the test were screened for non-cytopathogenic (ncp) pestivirus by PCR test and it was confirmed that there was no ncp pestivirus contamination. The continuous Madin-Darby Bovine Kidney (MDBK) cell line was used as a sensitive cell line to generate *EV-E*. MDBK cells and virus replication processes were performed using Modified Eagle's Medium (DMEM, Gibco) in an incubator at 37°C and 5% CO_2. The medium was supplemented with 10% FBS, 1% antibiotic (penicillin-streptomycin (10,000 U/mL)), and 1% L-glutamine for the cell generation processes.

For the neutralization assay, the *EV-E* isolate was inoculated into MDBK cell lines that had been passaged 24 hours ago and had a monolayer greater than 80% using the adsorption virus cultivation method. At the end of virus cultivation, cells were maintained in an incubator with 5% CO_2 at 37°C for 1 hour. At the end of the incubation period, the virus suspension in the cells was removed, and the inoculated cell lines were returned to the incubator after a cell production medium containing 2% FBS was added to the medium. Cells that showed a cytopathic effect of 80% or more under the tissue culture microscope were placed in a -30°C freezer and frozen/thawed. The resulting virus suspensions were transferred to sterile 15 ml centrifuge tubes. The virus suspensions were centrifuged at 3000 rpm for 5 minutes in a refrigerated centrifuge at +4°C. At the end of the procedure, the supernatant was transferred to cryovials as 1 ml. Portioned *EV-E* was stored in a freezer at -80°C for use in the study.

Virus titration test

To determine the infectious dose of *EV-E* used in the assay, a 50% tissue culture infectious dose analysis ($DKID_{50}$) was performed, and the titers of the virus were determined by this method. For this purpose, the virus suspension grown in stock was diluted tenfold with DMEM containing 2% FBS. (Sigma-Aldrch) Virus suspensions diluted in log 10 base were transferred to 96-well plates (TPP, Switzerland) in quadruplicate 100 µl volumes. Then, 50 µl of a cell suspension containing $5x10^4$ MDBK cells was added to each well. The plates were incubated in an incubator (Thermo, Scientific) with 5% CO_2 at 37°C for 72 hours. At the end of this period, the plates were examined under a tissue culture microscope (Olympus, Japan) for the formation of cytopathic effects (CPE) and the amount of $DKID_{50}$ per ml was calculated according to the Spearman-Karber (Spearman, 1908; Kärber, 1931) method (Figure 1).

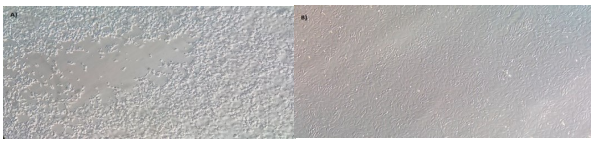

Figure 1. The cytopathic effect seen in titration test virus control. A) Virus control (CPE seen 24 h after seeding *EV-E* into MDBK cell line. B) Cell control (x60).

Serum neutralization test

For this study, pre-portioned serum samples were diluted and used as multiples of 3 and 5, considering the possibility of toxic effects that the sera might cause in cell culture. Each diluted serum was transferred to 96-well plates in 2 copies in 50 µl volume. The viral suspension, whose $DKID_{50}$ was known by the viral titration assay, was diluted to $100DKID_{50}$ with DMEM medium containing 2% FBS. 50 µl volume of 100 $DKID_{50}$ µl of virus suspension was transferred to wells containing 50 µl serum sample in a 1:1 ratio. At the end of the process, the study plates were incubated in an incubator with 5% CO_2 at 37°C for 1 hour. At the end of this period, 50 µl of cell suspension containing $5x10^4$ MDBK cells was added to the wells of the plates. The plates were transferred to an incubator with 5% CO_2 at 37°C. At the end of the 72-hour assay period, results were evaluated based on CPE formation in the cell culture.

Serum neutralization 50 Test (SN_{50})

The serum neutralization 50 (SN_{50}) assay was performed to determine antibody titers in positive samples in the serum neutralization assay. For this purpose, Ab (+) serum samples were diluted on a $\log_2$ basis, starting with 1/4 and 1/5 dilutions. The samples diluted on a $\log_2$ basis were transferred to 96 well plates as two copies in 50 µl volume, and the same volume of *EV-E* suspension was added at a ratio of $100DKID_{50}$. After virus addition, plates were transferred to an incubator with 5% CO_2 at 37°C and incubated for 1 hour. At the end of the incubation, 50 µl of the cell suspension containing $5x10^4$ MDBK cells was added to the wells of the plates. The plates were transferred to an incubator with 5% CO_2 at 37°C and incubated for 72 hours. The results were evaluated

for the presence of CPE at the final dilution point, and the antibody titer was determined.

Results

The serum samples of 504 calves of animals not vaccinated against *EV-E* in the Konya province were analyzed for *EV-E* antibodies. The result of this study was that 342 animals showed Ab(+) against *EV-E*; Ab (-) was detected in 162 animals (Figure 2). The rate of *EV-E* seropositivity in these tested animals was 67.85%, as shown in Figure 2; the seronegative animal rate was 32.14%.

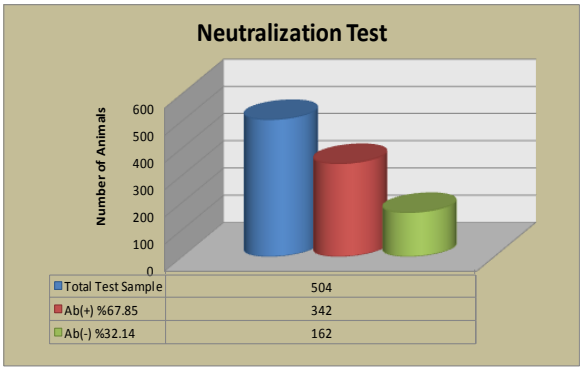

Figure 2. *EV-E* neutralization test results of samples.

When SN_{50} antibody titers of the seropositive animals were examined, in the 342 Ab(+) sample, neutralizing antibody titers of 1/4 (2.63%) in 9 animals, 1/10 (21.05%) in 72 animals, 1/15 (18.42%) in 63 animals, 1/20 (18.42%) in 63 animals, 1/24 (13.15%) in 45 animals, 1/30 (5.6%) in 18 animals, 1/48 (2.63%) in 9 animals, 1/60 (7.89%) in 27 animals, 1/64 (5.26%) in 18 animals, 1/80 (2.63%) in 9 animals, and 1/120 (2.63%) in 9 animals were observed (Figure 3). The analysis of the results shows that the most frequent neutralizing antibody titers in the calf population in the Konya province were 1/10, 1/15 and 1/20. Moreover, the highest antibody titers were defined between 1/80 and 1/120, and the total number of animals with the values reported in the study was 18 (Figure 3).

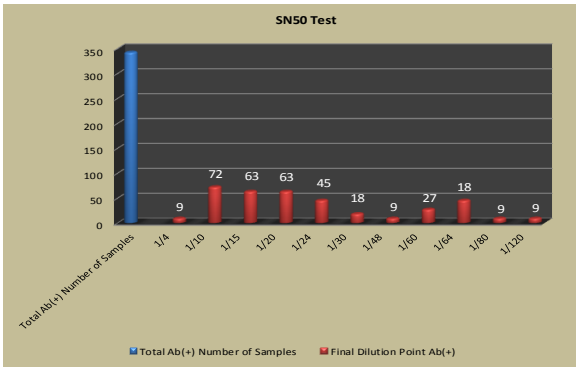

Figure 3. *EV-E* SN_{50} test results of samples.

Discussion and Conclusion

Bovine enteroviruses (BEVs) were first isolated in the late 1950s from the feces of cattle with symptoms of pneumonia, respiratory disease, enteritis, dysentery, and infertility, from fetal fluids of aborted calves, from the feces of healthy-appearing animals, and treated sewage (Li et al., 2012).

The present study investigated the seroprevalence of *EV-E* infection in calves in Konya province. For this purpose, serum samples from calves in the Konya province that had not been vaccinated against *EV-E* were tested for *EV type E* specific antibodies by neutralization assay. 342 samples were found to be seropositive. The studied population's seropositivity rate was 67.85% (Figure 2). In addition, neutralizing antibody titers of seropositive animals were evaluated with the SN_{50} test (Figure 3).

There are also studies in different countries on the seroprevalence of BEV. Gras et al. (2017) performed a neutralization test on 415 serum samples from cattle in Brazil and found that 411 samples (99.2%) had neutralizing antibodies to BEV. This study also found that 91 (22.1%) of the BEV seropositive samples had a high neutralizing antibody titer (1/320).

In another study investigating the prevalence of neutralizing antibodies to *BEV* in Korea, antibodies against *EV-E* and *EV-F* were evaluated in many living species, including cattle. As a result of the findings, the antibody against *EV-E* was 48.3%; ıt was shown that the antibody against *EV-F* was 68.1%. Neutralizing antibodies to these viruses were widespread in Korea, endemic in cattle, and the prevalence of antibodies to *EV-E* was lower than that to *EV-F* in cattle (Park et al., 2009). In a study conducted in China, the results of the serum neutralization test showed that two of nine bovine serum samples from naturally infected cattle had a neutralization capacity of $4^{2.66}$ and $4^{3.66}$, while the other seven bovine serum samples did not (Zhang et al., 2014). In a serological study investigating *BEV* circulation in llama and cattle populations in Argentina, specific antibodies against *BEV* were detected in 18 (4.10%) of 390 llama serum samples and 134 (91.78%) of 146 cattle serum samples. The rate was quite high (Puntel et al., 1999). Enteroviruses can infect different animal species and may be closely related genetically. In an enterovirus seroprevalence study conducted on 795 serum samples taken from wild opossums in New Zealand, it was found that opossums living in pastures where cattle and sheep graze did not have a higher prevalence of antibodies compared to animals living in forests. In the same study, opossum-derived enteroviruses were found to be genetically related to bovine enteroviruses (Zheng et al., 2010).

In a study by Alkan et al. (1997), in which 480 blood

serum samples were tested for BEV antibodies by neutralization assay, they found *EV-E* in 257 sera (53.5%) and *EV-F* in 233 sera (50.5%). Thus, this study was the first to demonstrate the presence of *EV -E* and *EV-F* infections in Türkiye. In another seroprevalence study conducted in Türkiye, specific antibodies against *EV-E* were detected for the first time in horses, dogs, goats, sheep, and humans. The highest prevalence was found in cattle at 64.8% (Gür et al., 2008). In a study on the seropositive rates of *EV-E* in camels in Türkiye, 92 blood serum samples were examined by neutralization test. As a result of the test, it was found that 30 (32.61%) of 92 serum samples were positive for *EV-E* (Erol et al., 2020). BEV can easily co-infect with other viruses that cause pathogenic infections in cattle, resulting in significant economic losses in the cattle industry (Luo et al., 2023). Although studies on infections caused by enteroviruses have been conducted in various animal species worldwide and in Türkiye, they have mainly focused on cattle. Previous studies have shown that *EV-E* infection, in particular, is quite common. In a study conducted in the provinces of Afyonkarahisar (Olukpınar, Emirdağ, Anıtkaya, Peribacaları), Eskişehir and Nevşehir, 1380 clinically healthy goat serum samples collected at 6 points and showing respiratory and reproductive problems were serologically tested for *EV-E* infection. 1380 goat serum samples showed a positivity rate ranging from 17.6% to 80% on a herd basis. 304 (41.8%) of 727 samples from Afyonkarahisar, 38 (53.5%) of 71 samples from Nevşehir, and 394 (67.7%) of 582 samples from Eskişehir were determined to be seropositive for *EV-E* in goats. It was found that the number of *EV-E* specific antibodies was significantly higher in herds with respiratory (593/1040) (57%) and reproductive (496/834) (59.4%) disorders compared to healthy animals (3/17) (17.6%). The result of the statistical analysis shows that there is a significant difference between healthy and diseased herds (Acar and Gür, 2009). On the other hand, Birdane and Gür (2019) obtained blood sera from a closed system intensive care dairy farm in Afyonkarahisar province, Central Anatolia region, in their serological studies for *BEV*. The samples were collected simultaneously shortly after birth. In the study, they preferred the virus neutralization test due to its high sensitivity and specificity. Serum samples were collected simultaneously from 155 calves at birth and their pre-colostral calves over three months. The test results showed that 98.7% (153/155) of calving cattle were seropositive for *EV-E*. The mean antibody titer (Ab) was the highest at 1/20 dilution. Specific antibodies were detected in 8 (5.1%) of 155 pre-colostral calves, and the distribution of Ab titers in these calves ranged from 1/5 to 1/40. In addition, the highest antibody titer values in these sera were found in calves at 1/10 and 1/80.

Comparing the current study with other studies conducted in Türkiye (Alkan et al., 1997; Gür et al., 2008; Acar and Gür, 2009), we find that the *EV-E* seroprevalence rate is similar. At the same time, these results show that the virus is widely distributed in the field. In the study of Gür et al. (2008), the highest seropositivity value among eight animal species (horse, sheep, goat, cattle, dog, human, gazelle, camel) was found in cattle (986/1520) (64.8%), sheep (84/281) (29.8%) and goats (132/477) (27.6%). These reported data indicate that enteroviruses are more prevalent in ruminants.

Since BEV is transmitted via the fecal-oral route and has a broad host range, hygiene and biosecurity programs for disease prevention should be carefully followed. In examining immunization trials around the world, there is currently no vaccine that prevents BEV infection. Nevertheless, a study conducted in China highlighted that it is a good candidate for a viral vaccine vector (Ren et al., 2020). Especially due to the limited number of immunization studies on *BEV*, it is important to increase and report research in this area.

Therefore, it is suggested that the differences in seropositivity rates determined in the sampled areas may be caused by many factors, such as the differences in the number of animals in the sampled population and farms, the differences in husbandry conditions, the type of rearing, the timing of infection, the age distribution of the animals, etc. This study indirectly detected the presence of *EV-E* infection in Konya province. Moreover, the high seroprevalence detected indicates that the importance of *EV-E* in the Konya province may gradually increase. It is important to investigate further the epidemiology of infections caused by bovine enteroviruses, especially to study the impact of clinical features of infection on livestock, identify the genotypes circulating in the country, and study their phylogenetic structures.

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