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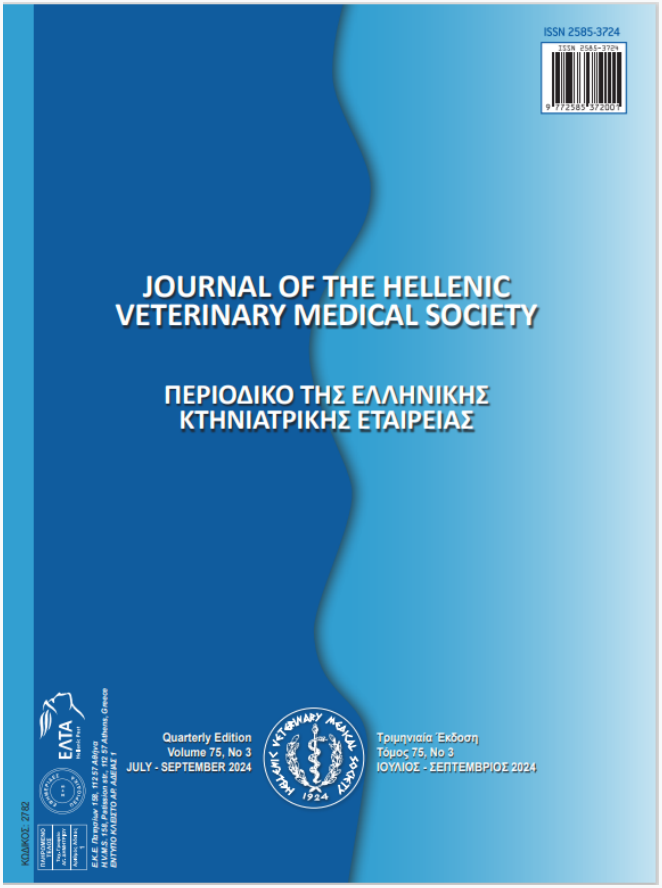

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Detection of viral infectious agents causing calf diarrhea on dairy farms in Serbia

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ABSTRACT: Neonatal calf diarrhea (NCD) is a multifactorial disease commonly occurring in unweaned calves, and one of the major causes of calf losses worldwide. In this study, we investigated the role of viral agents in neonatal calf diarrhoea from dairy farms with reported outbreaks in the winter of 2021-2022. For this study, we tested rectal swab samples from 20 commercial dairy farms with 100-1000 cows with reported acute diarrhoea in neonatal calves. From each farm, five to ten calves, up to 2 months of age, in total 154, with acute diarrhea were sampled by rectal swabbing. Out of 20 tested dairy farms, at 4 farms none of the tested viruses was detected whereas at least one of the tested viruses was detected at 16 farms (80%). As the most prevalent, mono BRV infection was detected at 10 farms (62.5%), followed by a single BCoV infection at 3 farms (18.75%). Coinfection of BVDV and BRV was demonstrated at 2 farms (12.5%), and simultaneous infection with BVDV, BRV and BCoV at one farm (6.25%). G typing of BRV revealed that 60% of the BRVs were G10, 33.3% were G6, and 16.7% were G8. P typing indicated that 66.7% of the BRVs were P[11] and that 33.3% were P[5]. This study revealed viral causative agents being the most often found in diarrheic neonatal calves in Serbia, thus contributing to the development of more effective strategies for disease prevention and control.

Keywords: Bovine Rotavirus; Bovine Coronavirus; Bovine Viral Diarrhea Virus; calf neonatal diarrhea; Serbia

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INTRODUCTION

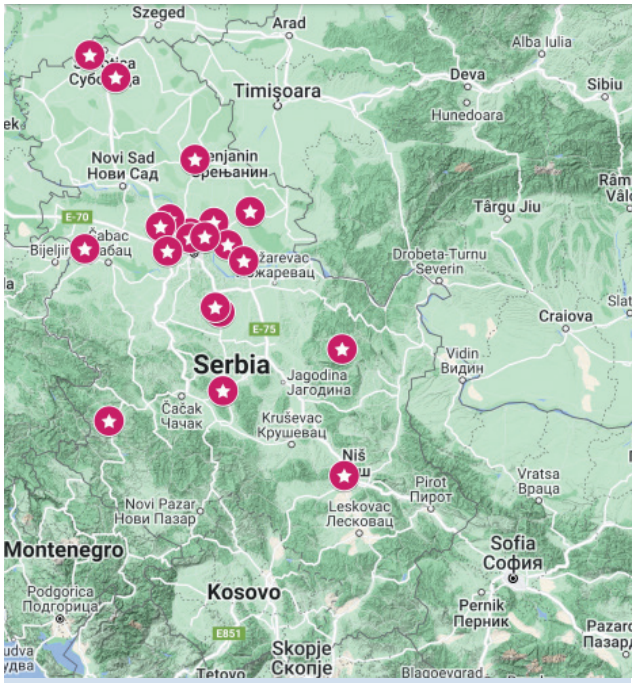
Neonatal calf diarrhea (NCD) is a multifactorial disease commonly occurring in unweaned calves, and one of the major causes of calf losses worldwide. The disease is primarily caused by infectious agents, such as viruses, bacteria, and protozoa (Cho and Yoon 2014), rarely as mono-infection, but more often as co-infection of several pathogens (Gomez and Weese 2017). Additionally, non-infectious factors including farm management and herd size significantly influence the occurrence and severity of the disease (Cho and Yoon, 2014). The most frequently reported viruses that cause calf diarrhea are Bovine Rotavirus (BRV), Bovine Coronavirus (BCoV), and Bovine Viral Diarrhea Virus (BVDV) (Cho and Yoon 2014). Depending on the age, BRV infection is commonly seen in calves old up to 14 days (Brunauer et al. 2021), while BCoV is considered a cause of diarrhea in calves up to one month of age (Cho and Yoon, 2014). BVDV infection manifests with various clinical signs, occasionally causing calf diarrhea regardless the age (Goto et al. 2021). Nevertheless, many other viruses, such as torovirus, parvovirus, norovirus, kobuvirus, adenovirus (Lee et al. 2019), astrovirus (Turan and Isidan, 2018), etc. are recognized as enteric pathogens and isolated from diarrheic calves. Generally, viral calf diarrhea is characterized by voluminous, liquid feces often with large amounts of mucus, while the mortality rate riches 58% (Azizzadeh et al. 2012). Besides direct economic losses from mortality, substantiate losses are indirect from the cost of medication, labour needed to treat sick calves, delayed growth of calves, and higher age at first calving (Gomez and Weese, 2017). Given that diarrhea is a leading cause of sickness and death of calves, field investigations of outbreaks should be thorough enough to enable the reduction of the losses through treatment, control and prevention of new cases, supported by an accurate laboratory diagnosis (Smith 2012). For the etiological diagnosis, fecal samples from untreated calves should be collected but taking into account that yet after 12 to 18 hours of the onset of clinical signs the value of a fecal sample diminishes rapidly. Most commonly, nucleic acid-based techniques, such as PCR, and an antigen-capturing enzyme-linked immunosorbent assay (AgELISA), are used for the detection of different pathogens in clinical specimens from diarrheic calves. Neonatal calf diarrhea can be prevented and controlled using vaccination (Uetake 2013). However, biosecurity aiming at reducing both likelihood of the introduction of an infection agent and its transmission is of utmost importance (Barrington et al. 2002).

Following reports from several dairy farms with acute diarrhea outbreaks during the wintertime 2021-2022, we decided to investigate the role and contribution of viral infectious agents in neonatal calf diarrhea in Serbia.

MATERIAL AND METHODS

Sampling

For this study, we tested rectal swab samples from 20 commercial dairy farms with 100-1000 cows with reported acute diarrhoea in neonatal calves. The animals were reportedly vaccinated against BRV and BCoV at 13 farms. Rectal swabs were taken from five to ten calves, up to two months of age, with reported symptoms of acute diarrhoea from each farm. In total 154 rectal swabs were collected. The location of the farms are shown on the map 1.



Map 1: Locations of the farms where the study was carried out

Virological analysis

Rectal swabs were immersed into 1 ml of sterile PBS and thoroughly vortexed. The suspensions were centrifuged for 10 min at 4.000 rpm and decanted supernatants were used for RNA extraction (IndiSpin *Pathogen Kit*, Indical, Germany). The samples were tested for the most common viral causal agents of diarrhea in cattle, namely BVDV, BRV and BCoV. Real-time RT-PCR was used for the genome detection of BVDV and BCoV, while gel-based RT-PCR was

Table 1: The primers` and probes` nucleotide sequences used for the genome amplification of BVDV, and BCoV

Name	5` - 3` sequence	Target region	Reference
BCoV-F	CCTTCATATCTATACACATCAAGTTGTT	M gene	[11]
BCoV-R	ACCAGCCATTTTAAATCCTTCA		
BCoV-Pb	FAM - CCTTCATATCTATACACATCAAGTTGTT - TAMRA		
BVD 190-F	GRAGTCGTCARTGGTTCGAC	5`UTR	[12]
V326	TCAACTCCATGTGCCATGTAC		
TQ-pesti	FAM - TGCYAYGTGGACGAGGGCATGC - TAMRA		

carried out for BRV detection. Real-time RT-PCR was completed using a commercial kit, Luna® Universal One-Step RT-qPCR Kit (NEB, USA). The reaction mix was composed of 2.5 µl template RNA, 6.25 µl Luna Universal One-Step Reaction Mix (2x), 0.63 µl of 20x Luna WarmStart RT Enzyme Mix (20x), 0.5 µl of each primer (10 µM), 0.25 µl of probe (10 µM) and 1.88 µl Rnase-free water. The primers` and probes` nucleotide sequences are given in table 1.

The reactions were completed throughout the following steps: reverse transcription at 55 °C for 10 min, initial denaturation at 95 °C for 1 min, and 40 cycles of denaturation at 95 °C for 10 sec, annealing-extension at 60 °C for 30 sec.

Gel-based RT-PCR for the detection of BRV genome was completed using QIAGEN OneStep RT-PCR Kit (Qiagen, Germany) and primers VP6-F 5′-GACGGVGCRACTACATGGT-3′ and VP6-R 5′-GTCCAATTCATNCCTGGTG-3′) amplifying 379-bp region of the VP6 gene (Mukhopadhya et al. 2013). The reaction mixture was composed of 4 µl 5x QIAGEN OneStep RT-PCR Buffer, 4 µl 5x Q-Solution, 0.8 µl dNTP Mix, 0.8 QIAGEN OneStep RT-PCR Enzyme Mix, 1.2 µl of each primer (10 µM), 6 µl RNase-free water, and 2 µl template. The thermal cycling protocol was as follows: reverse transcription at 50 °C for 30 min, initial denaturation at 95°C for 15 minutes, followed by 40 cycles of denaturation at 94°C for 1 min, primers annealing at 55°C for 1 min and extension at 72°C for 1 min, followed by final elongation at 72 °C for 10 min. PCR products were analyzed in a 2% agarose gel stained by ethidium bromide and visualized under UV light after electrophorese at 60 V for 1 hour. Determination of P and G serotypes of BRV from positive samples was performed by a nested RT-PCR typing assay as described by Falcone *et al.* (1999). In brief, after the full length of VP7 amplification, G6, G8, and G10-specific G-typing primers were used in the second round of PCR. For P serotyping, in the first round, the partial VP4 gene was amplified, while P[1], P[5], and P[11] serotype-specific primers were used in the second amplification.

For the statistical analysis, one-way ANOVA test for independent measures and Chi-square were applied.

RESULTS

Out of 20 tested dairy farms, none of the tested viruses was detected at 4 farms whereas at least one of the tested viruses was detected at 16 farms (80%) (Table 2).

As the most prevalent, mono-BRV infection was detected at 10 farms (62.5%), followed by a single BCoV infection at 3 farms (18.75%). Coinfection of BVDV and BRV was demonstrated at 2 farms (12.5%), and simultaneous infection with BVDV, BRV and BCoV at one farm (6.25%). On farm B, 2 calves were found positive for both BVDV and BRV, and one for BCoV and BRV. On farm H, one calf was positive for both BVDV and BRV. The average percentage of positive calves was 26.6% for BVDV, 45.35% for BCoV, and 79.7% for BRV.

Four farms that used vaccination as a control measure against viral calf diarrhea were negative for the presence of the three most common viruses whereas a single BRV infection was discovered on all 5 farms where vaccination was not practiced.

Considering the results of one-way ANOVA test (f-ratio value is 16.6, p-value is <0.00001; p <0.05), BRV infection is significantly more prevalent than the BVDV and BCoV infections. However, significant differences between vaccinated and unvaccinated farms were not observed (p-value is 0.7; < 0.05).

G typing revealed that 60% of the BRVs were G10, 33.3% were G6, and 16.7% were G8. P typing indicated that 66.7% of the BRVs were P[11] and that 33.3% were P[5]. Serotype P[1] was not detected, while one

Table 2: Summary of obtained results per farm

FARM	No. of tested calves	No. positive calves (%)			Vaccination (Y/N)	Status
		BVDV	BRV	BCoV		
A	7	0	0	5 (71.4)	Y/BRV, BCoV	BCoV
B	10	2 (20)	8 (80)	2 (20)	Y/BRV, BCoV	BVDV, BRV/G8P[11], BCoV
C	9	0	9 (100)	0	N	BRV/G6P[5]
D	8	0	5 (62.5)	0	N	BRV/G10P[11]
E	7	0	7 (100)	0	N	BRV/G10P[11]
F	5	0	3 (60)	0	N	BRV/G10P[11]
G	6	0	2 (33.3)	0	N	BRV/G10P[5]
H	10	4 (40)	10 (100)	0	Y/BRV, BCoV	BVDV, BRV/G10P[11]
I	10	0	6 (60)	0	Y/BRV, BCoV	BRV/G6P[5]
J	10	0	0	0	Y/ BRV, BCoV, BVD	negative
K	8	0	0	0	Y/ BRV, BCoV, BVD	negative
L	10	0	0	0	Y/BRV, BCoV	negative
M	9	0	0	0	Y/ BRV, BCoV, BVD	negative
N	5	0	0	3 (60)	Y/BRV, BCoV	BCoV
O	8	0	6 (75)	0	Y/BRV, BCoV	BRV/G10P[11]
P	7	0	7 (100)	0	Y/BRV, BCoV	BRV/G6P[5]
Q	5	1 (20)	5 (100)	0	Y/BRV, BCoV	BVDV, BRV/not typed
R	10	0	0	3 (30)	Y/BRV, BCoV	BCoV
S	10	0	8 (80)	0	Y/BRV, BCoV	BRV/G6P[11]
T	7	0	6 (85.7)	0	Y/BRV, BCoV	BRV/G8P[11]

BRV could not be serotyped. Several P and G combinations were discovered whereasG10P[11] was found most frequently (41.7%), followed by G6P[5] (25%). With regard to vaccination, G10P[11] was the most prevalent (60%) at unvaccinated farms. However, at vaccinated farms, G10P[11], G6P[5], and G8P[11] were equally represented at 28.6%, while G6P[11] was discovered at one farm (14.3%).

DISCUSSION

Neonatal diarrhea in calves (NCD) is the leading cause of morbidity and mortality in calves. Several infectious agents have been reported as major causes of it, additionally supplemented with immunity issues, environmental stress and contamination (Cho and Yoon, 2014). Furthermore, NCD may not be necessarily related to infections, as shown that iron deficiency is also a predictor of the disease (Prodanovic et al. 2019). However, to be able to apply suitable preventive and mitigating measures, etiological diagnosis is of utmost importance. Reports and researches from Serbia related to neonatal calf diarrhea are rather scarce. Therefore, in this study, we focused on viral pathogens, and their incidence in calves up to 30 days of age. However, the bias of obtained results due to the sampling procedure cannot be excluded. In this study, it was found that 80% of farms facing acute neonatal calf diarrhea were diagnosed with one or more viral infections, whereas mono-infection with BRV was highly prevalent, as found at 62.5% of farms. Rotaviral infection was followed by mono-BCoV, BVDV-BRV, and BVDV-BRV-BCoV co-infections. Additionally, BRV infection was the most prevalent at the herd level, where up to 100% of tested calves were found positive. These results are in correlation with findings communicated by other authors who reported that the majority of BRV-BCoV infections were identified in Europe, while the highest prevalence of BRV-ETEC and BRV-Crypto were determined in West Asia (Brunauer et al. 2021). Further to coinfection at farms, interestingly, concurrent infections of calves with 2 or 3 viral pathogens were also confirmed, namely BVDV and BRV in three calves, and BRV and BCoV in one calf. Due to the immunosuppression effect, and the persistent infection, BVDV infection was reported together with other pathogens (Yesari et al. 2021). Besides, it has been shown that, causing villus atrophy in the duodenum and submucosal inflammation of the intestines, BVDV promotes effects of BRV, thus causing more severe clinical manifestation in concurrent infection with BVDV and BRV than infection with BRV or BVDV alone (Kelling et al. 2002). Similarly, triggering immune impairment, and enhancing the replica-

tion of other viral agents, BCoV has been found in concurrent infections with BRV (Atasoy et al. 2022), as well as BVDV (Niskanen et al. 2002). Given the diversity of strains and considering its epidemiologically importance, detected BRV isolates were further serotyped. Though many different combinations of G and P serotypes in cattle have been found, only G6, G10 and G8 combined with P[5], P[11], and P[1] are of epidemiological interest. Similarly like in the Americas, Europe, Asia and Australia (Ahmed et al. 2022), G10 and G6 serotypes, as well as P[11] and P[5] were the most frequently detected in this study. Also, in relation to the G and P combinations, represented at 66.7%, G10P[11] and G6P[5] were by far the most prevalent BRVs like in many other countries(Papp et al. 2013).

NCD is mainly related to the calving season, depending on the farm management. However, it seems that seasons have a significant effect on the occurrence of diarrhea thus making rainy months had a higher incidence of the disease (Monney et al. 2020). The presented results, investigating the outbreaks exactly during the cold months are supportive to these findings. This is not surprising given that wet and cold environmental conditions weaken the immune system enabling the pathogens to cause clinical disease. Therefore, it is recommended to adjust the calving season to favourable weather conditions (Cho and Yoon, 2014). In relation to mitigation and prevention, biosecurity, both inner and outer, is of utmost importance. In the case of viral diarrhea given that the transmission occurs predominantly by fecal-oral route, hygiene on the farm is considered as essential to reduce and limit its spread. Even more, hygiene is directly linked to the infectious dose that further determines the severity of clinical symptoms. Generally, it is considered that larger farms have biosecurity at a higher level than small ones (Sahlström et al. 2014). On the contrary, small, family farms have better colostrum management and/or quality that provides calves with the higher IgG levels that are key for the protection after the birth (Barry et al. 2019). In this study, samples originated from commercial farms, but with no data on biosecurity. However, recent results (Samolovac et al. 2021) showed that, in Serbia, the greatest risks and threats to biosecurity were manifested exactly at the earliest age of calves, in particular concerning the colostrum uptake and pathogens exposure. Additional to the good farm management, including hygiene and nutrition, vaccination of dams or calves could be used for the prevention of NCD, as shown in this study where 75% of farms used active immunization to control it. However, regarding the BRV infection, there were no significant differences between vaccinated and unvaccinated farms. Vaccination is generally considered effective against predominant BRV strains, but there is no efficacious cross-protection to different genotypes (Liu et al. 2021). Given that vaccinated farms in this study used a vaccine composed of G6P[5], the occurrence of NCD caused by other serotypes was not surprising. The incursion of G10P[5] which is the most prevalent at unvaccinated farms and high infectious pressure, as well as, G8P[11], should be taken as an alert and considered for inclusion in vaccine compositions. This should indicate the necessity for the assessment of biosecurity measures and their weaknesses, and further applied for the prediction of routes for the introduction of other pathogens on the farm. Only at two vaccinated farms, G6P[5] was confirmed, probably because of a laps in correct vaccination procedures. Though the benefits of vaccination are indisputable, surveillance of rotavirus G and P genotypes should be regularly practiced at cattle farms. This would allow better implementation of preventive measures and correct vaccine selection. The same principle should be generally applied since the assumption of etiological diagnosis of neonatal calf's diarrhea based on the clinical signs is difficult and unreliable, but curtail for implementation of mitigation measures.

This study revealed viral causative agents being the most often found in diarrheic neonatal calves in Serbia, thus contributing to the development of more effective strategies for disease prevention and control.

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