Opis przypadku *Case report*

A case of acute respiratory disease associated with Bovine Coronavirus (BCoV) infection in calves at a cattle farm in eastern Poland

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Summary

Bovine Respiratory Disease Complex (BRDC) and Neonatal Calf Diarrhea (NCD) are the two most important syndromes responsible for the majority of mortality cases in calves. They have a complex etiology with multiple viral agents involved, including Bovine Coronavirus (BCoV). Respiratory and enteric signs were observed in 11 calves newly introduced into a herd in eastern Poland. Within two weeks, eight fatal cases were noticed. Results of an on-site antigenic serological test performed by a veterinary surgeon on one of the calves, pointed to a possible BCoV infection. To confirm involvement of this virus, samples from seven affected animals were subjected to testing using real time RT-PCR and RT-PCR assays specific to BCoV. To exclude other potential respiratory and enteric viral pathogens, additional real time PCR tests were performed for Bovine Viral Diarrhoea Virus (BVDV), Bovine herpes virus – 1 (BoHV-1) and Bovine respiratory syncytial virus (BRSV). Genetic material of BCoV was detected in all tested animals by RT-PCR and in 6 out of 7 by real time RT-PCR, while no samples were positive for BVDV, BoHV-1 or BRSV. Sequence analysis of BCoV isolates showed their close relation to each other and to the Polish strains identified in recent years. Our results confirmed that BCoV could be a causative factor of fatal cases in calves even in absence of other viruses involved. They also point to the need to quarantine calves before introduction into the herd, and monitor their health.

Keywords: calves, BRDC, NCD, BCoV

Keeping newborn calves in good health is important for the economy of cattle farming. Diseases generate both direct costs associated with mortality and treatment but also indirect costs connected with decreased growth and performance (14). Mortality in calves is mainly associated with cases of enteric diseases (around 40-50% of deaths) or respiratory diseases (around 15% of cases). In both disease complexes, multiple factors are involved, including infectious agents (viral, bacterial and/or protozoal) as well as individual host susceptibility and environmental factors (3, 15, 18, 24). One of the viral infectious agents of major importance, known to be involved in both Neonatal Calf Diarrhea (NCD) and Bovine Respiratory Disease Complex (BRDC) is Bovine Coronavirus (BCoV). Bovine Coronavirus is an enveloped RNA virus belonging to the *Betacoronavirus* genus within *Coronaviridae* family. While it remains controversial whether BCoV could cause respiratory disease alone since experimental infections showed equivocal results, its role as an important primary pathogen causing NCD is widely recognized (10, 20). Some studies estimate that BCoV could be responsible for as much as one third of the cases of diarrhea in calves, causing more severe pathological changes in the digestive tract than rotavirus infections (5). In Poland, BCoV infections are common among cattle, with most recent study from 2022 showing 70.6% seroprevalence and 10.5% of infected animals (19). While no fatalities were described in that study, a correlation between signs of respiratory disease and infection with BCoV was observed. In the current study, we described multiple fatal cases in a cattle herd in Poland among newly introduced calves with respiratory and enteric diseases that could be associated with infections with BCoV.

Material and methods

Animals. Eleven 2-3 week old calves of mixed breed were introduced to a cattle farm located in the Mazowieckie region. The animals originated from a breeding farm in southern Poland (Małopolska region), and their immunological status for major cattle pathogens was unknown prior to the shipment to the new herd. Disease symptoms were observed one day after introduction to the herd. These included: apathy, reduced appetite, lethargy, inflammation of the upper respiratory tract, and watery diarrhea. Body temperature varied between 37.5°C and 39.5°C. In two severe cases, 5% glucose and multi-electrolyte solution were administered intravenously, with the addition of 2.5 mg tulathromycin and 2 mg of flunixin per kg of body mass. The remaining calves, received tulathromycin, electrolytes, linseed, and horse chestnut tannins. Despite these interventions, the first cases of mortality appeared after two days from initial symptoms. In total, from 11 calves, 8 died within two weeks of introduction to the herd. The surviving calves showed weak respiratory symptoms and lack of appetite. Autopsy of fallen animals showed intestinal congestion and inflammation accompanied by enlargement of mesenteric lymph nodes. Additionally, in some animals, inflammation of abomasum was observed. From one of the calves with diarrhea a blood sample was collected and tested on-site using REDTEST Professional BCV+Rotavirus+Giardia-Cryptosporidium antigenic test (Sigmed, Poland), which yielded a positive result for presence of BCoV antigen.

Samples. Fragments of lymph nodes, spleen and lung samples were collected from 5 fallen calves while nasal swabs were collected from 2 animals that survived the outbreak. Approximately 2 g fragments of each tissue were cut, and 10% homogenates were prepared in MEM using Ultra Turrax T25 (IKA Warke, Staufen, Germany). Collected dry nasal swabs were submerged in 1 ml of MEM medium and incubated for 30 minutes before further use.

DNA and RNA extraction. Genetic material was isolated from samples collected from all 7 animals included in the study: 5 fallen animals (tissue samples) and 2 survivors of the outbreak (nasal swabs). Viral RNA was extracted from 140 µL of nasal swab samples and tissue homogenates using a QIA amp Viral RNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's manual. Resulting RNA was eluted in 50 µl of an elution buffer and stored at –70°C for use in RT-PCR reactions.

Viral DNA was extracted from 200 µL of nasal swab samples and tissue homogenates using a QIA amp DNA Mini kit (Qiagen), eluted in 30 µL of elution buffer and stored at –70°C for use in PCR reactions.

Real time RT-PCR. Four real time PCR reactions were run, each specific to different viruses associated with respiratory or enteric diseases: BCoV, BoHV-1, BRSV and BVDV. For BVDV diagnostic, commercial real-time RT-PCR testsRealPCR BVDV RNA Mix (IDEXX, Montpellier, France) and RealPCR RNA Master Mix (IDEXX, Montpellier, France) were used. Reaction mix and temperature conditions were applied according to the manufacturer's manual. For control, internal positive control provided with the test was used.

For BoHV-1, the real time PCR reaction was run as previously described by Abril et al. 2004 (23). The reaction mix consisted of 14.5 µL of water, 5 µL of 5 × QuantiTect Virus Master Mix (Qiagen, Hilden, Germany), 1 µL of both BHVgB-F forward (4.5 µM) and BHV-gB-R reverse (4.5 µM) primers, 1 µL of BHV-gB-Pb probe (5 µM) and 2.5 µL DNA sample, for a total volume of 22.5 µL. The reaction was run starting with 5 min of incubation at 50°C followed by initial 2 min denaturation at 95°C. Next, 45 cycles of amplification were run, each consisting of 15 s of denaturation at 95°C and 45 s of annealing/elongation at 60°C. As a positive control, BoHV-1 strain Colorado (ATCC, VR-864) was used.

Bovine respiratory syncytial virus specific real-time RT-PCR was performed using previously designed primers and TaqMan probe specific to F protein gene of the virus described by Hakhverdyan et al. 2005 (12). The reaction mix consisted of 16.25 µL of water, 5 µL of 5 × QuantiTect Virus Master Mix (Qiagen, Hilden, Germany), 0.25 µL of both BRSV485F forward (10 µM) and BRSV569R reverse (4.5 µM) primers, 0.5 µL of BRSV probe (10 µM), 0.25 µL of 100 × QuantiTect Virus RT Mix (Qiagen, Hilden, Germany) and 2.5 µL RNA sample for a total volume of 22.5 µL. The reaction was run starting with 30 min of reverse transcription at 50°C followed by initial 3 min denaturation at 95°C. Next, 45 cycles of amplification were run each consisting of 15 s of denaturation at 94°C, 15 s of annealing at 50°C and 15 s of elongation at 72°C. As a positive control, BRSV strain 375 (ATCC, VR-1339) was used.

Bovine coronavirus specific real-time RT-PCR was performed using previously designed primers and TaqMan probe specific to M protein gene of the virus (8). For internal control, additional primers and probes specific to β-actin were used as described by Toussaint et al. 2007 (23). The reaction mix consisted of 6.3 µL of water, 4 µL of 5 × QuantiTect Virus Master Mix (Qiagen, Hilden, Germany), 2 µL of both BCoV-F forward (10 µM) and BCoV-R reverse (10 µM) primers, 2 µL of BCoV-Pb probe (5 µM), 1.5 µL of a mixture of primers and probes specific to bACT, 0.2 µL of 100 × QuantiTect Virus RT Mix (Qiagen, Hilden, Germany) and 2 µL of RNA sample, in total volume of 20 µL. The reaction was run starting with 30 min of reverse transcription at 42°C and a 10 min incubation at 95°C. Next 40 cycles of amplification were run each consisting of 15 s of denaturation at 95°C and 45 s of annealing/elongation at 58°C. As a positive control, for each PCR reaction, BCoV S379 Riems strain was used. All real-time PCR amplifications were performed using a LightCycler 96 Instrument (Roche, Mannheim, Germany). Information on primers and probes used in reactions (excluding commercial BVDV test) is shown in Table 1.

RT-PCR and Sanger sequencing. All RNA samples were amplified with a conventional RT-PCR using Sp1 and Sp2 primers specific to the conserved fragment of the S gene encoding the spike protein of BCoV with the Transcriptor One-Step RT-PCR kit (Roche, Mannheim, Germany) (8). The reaction mix was prepared in a total volume of 25 µL which included 1 µL of each primer (10 µM), 5 µL of 5 × reaction buffer, 0.5 µL of enzyme mix, 15.5 µL of PCR grade water and 2 µL of RNA sample. The amplification steps consisted of 30 min of reverse transcription at 50°C followed by 2 min of incubation at 94°C and 45 cycles consisting of 30 s of denaturation at 94°C, 30 s of annealing

Tab. 1. Primers and probes used for BRSV, BCoV, BoHV-1 and BVDV diagnostics using real time PCR tests

Specificity	Probe/Primer	Sequence (5'-3')	Target gene	Product size	Reference
BoHV-1	BHV gB-F	TGTGGACCTAAACCTCACGGT	gB	97	Abril et al. 2004 (1)
	BHV gB-R	GTAGTCGAGCAGACCCGTGTC			
	BHV-Pb	FAM-AGGACCGCGAGTTCTTGCCGC-TAMRA			
BCoV	BCoV-F	CTGGAAGTTGGTGGAGTT	M	85	Decaro et al. 2008 (8)
	BCoV-R	ATTATCGGCCTAACATACATC			
	BCoV-Pb	FAM-CCTTCATATCTATACACATCAAGTTGTT-BHQ1			
BRSV	BRSV-F-485F	AAGGGTCAAACATCTGCTTAACTA	F	85	Hakhverdyan et al. 2005 (12)
	BRSV-F-569R	TCTGCCTGWGGGAAAAAAG			
	BRSV-F-TaqMan-546	FAM-GAGCCTGCATTRTCACAATACCACCC-BHQ1			
BVDV*	–				–
	–	–	–	–	
	–				

Explanations: * Manufacturer of the Real PCR BVDV RNA Mix does not provide exact data about primer and probe sequences, product size and specificity of the test

at 55°C and 30 s of elongation at 68°C. The reaction was finished by a 10 min incubation at 68°C. Specific 622-nucleotide-long products were visualized by electrophoresis in 1.5% agarose gel. Positive samples were purified and used for Sanger sequencing with the same set of primers that were used for RT-PCR. Sanger sequencing was performed by Genomed SA (Warsaw, Poland). The resulting partial sequences of the S gene were aligned with other selected coronavirus sequences available in GenBank using MEGA 11 software, and phylogenetic tree was constructed using the neighbor-joining method (16).

Results and discussion

None of the tested samples were positive for BVDV, BRSV or BoHV-1 in real time PCR reactions. In contrast, presence of Bovine coronavirus infections was confirmed by real time RT-PCR in 6/7 animals and by classical RT-PCR in 7/7 animals. Discrepancy observed between results from both methods was surprising, especially as it was previously shown that among these two methods, sensitivity of real time RT-PCR is 10 times higher. However, the same authors described that one BCoV isolate remained undetected by this method while positive in classical RT-PCR. This was caused by mutation in fragment of M gene that prevented proper binding of the TaqMan probe (8). As the genetic analysis of M gene of BCoV isolates was not the aim of our study we cannot exclude that a similar situation appeared in the case of one of our isolates.

The virus was present in tissue and nasal swab samples from animals that successfully recovered from the disease and those that died as a result of infection (Tab. 2). In total, seven partial sequences of S (spike) gene of BCoV were acquired as a result of Sanger sequencing. Prior to analysis sequences were trimmed at both ends to eliminate fragments of poor quality. As a result, 573 bp long fragments were submitted to GenBank under accession numbers PX058828 – PX058833 and used for further analysis. Sequences

Tab. 2. Results of real time PCR reactions for BoHV-1, BVDV, BRSV and BCoV diagnostics. Samples were defined as positive for Ct < 40.0

Animal	BoHV-1		BVDV		BRSV		BCoV		Sample	Disease outcome
	Ct	result	Ct	result	Ct	result	Ct	result		
1	No Ct	–	No Ct	–	No Ct	–	No Ct	–	Tissue mix**	Dead
2	No Ct	–	No Ct	–	No Ct	–	29.02	+	Tissue mix	Dead
3	No Ct	–	No Ct	–	No Ct	–	30.13	+	Tissue mix	Dead
4	No Ct	–	No Ct	–	No Ct	–	20.02	+	Tissue mix	Dead
5	No Ct	–	No Ct	–	No Ct	–	26.75	+	Tissue mix	Dead
6	No Ct	–	No Ct	–	No Ct	–	19.91	+	NasalSwab	Recovered
7	No Ct	–	No Ct	–	No Ct	–	17.61	+	NasalSwab	Recovered
8	n/t*	n/t	n/t	n/t	n/t	n/t	n/t	n/t	–	Recovered
9	n/t	n/t	n/t	n/t	n/t	n/t	n/t	n/t	–	Dead
10	n/t	n/t	n/t	n/t	n/t	n/t	n/t	n/t	–	Dead
11	n/t	n/t	n/t	n/t	n/t	n/t	n/t	n/t	–	Dead

Explanations: * Not tested; ** Mixed homogenates of lymph nodes, spleen and lungs

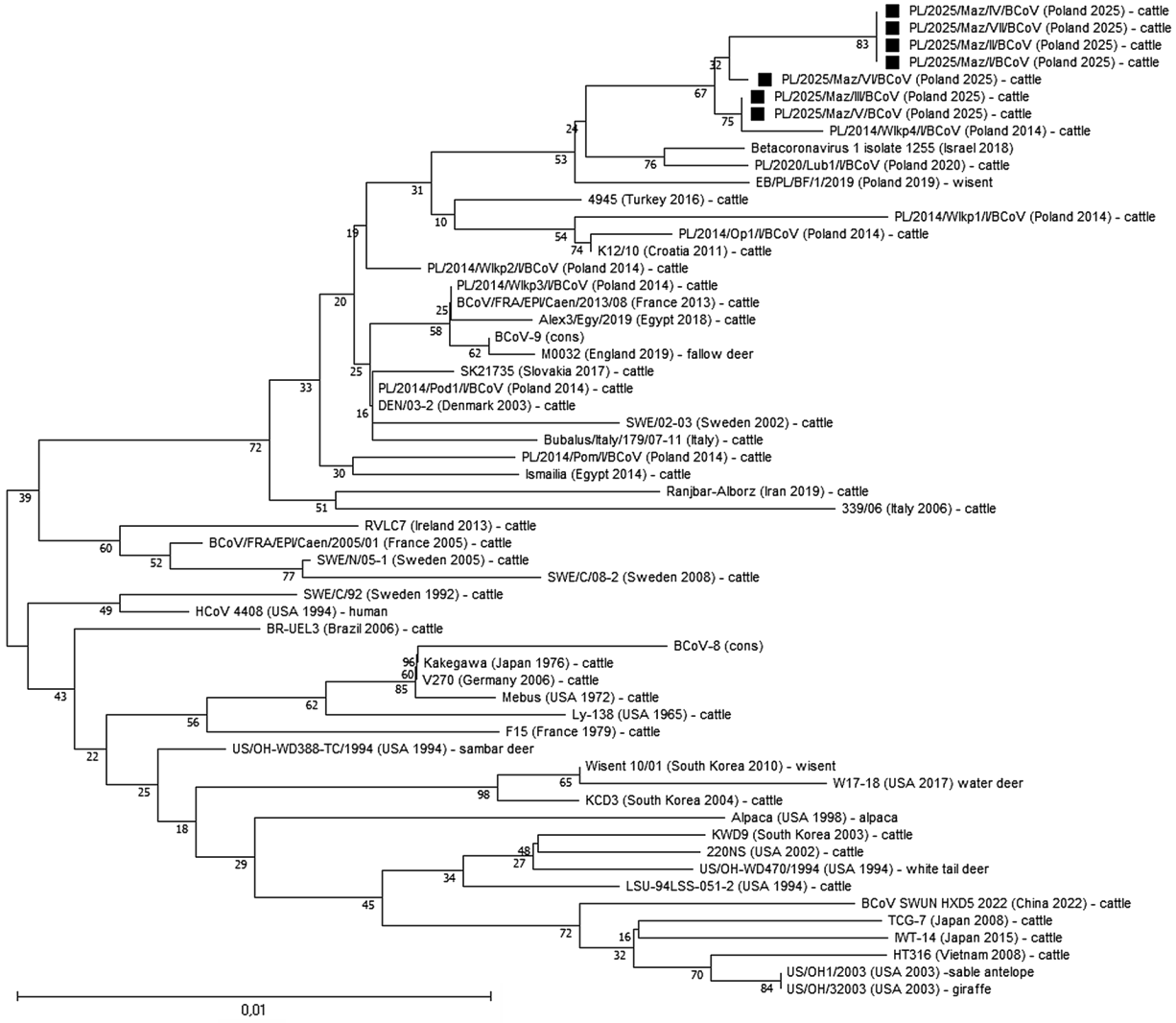

Fig. 1. Neighbor-joining phylogenetic tree of BCoV constructed based on the 573 nucleotide long sequence of S gene. Sequences acquired in this study are marked by black square (■). Information about host, country of origin, and date of sample collection is included next to the name of each strain

were aligned with each other and with previously identified BCoV strains using MEGA11, and a phylogenetic tree was constructed by the neighbor-joining method (Fig. 1) (16).

Sequence analysis showed that strains identified in the study exhibited a very high degree of nucleotide sequence homology to each other spanning 99.65--100%, and with the PL/2014/Wlkp4/I/BCoV strain, isolated in western Poland in 2014, at 99.48-99.83% (19). This indicates that identified strains were most probably of local origin and not introduced to the country by recent import of infected animals. This result was similar to what was observed in Sweden, where high genetic identity of BCoV isolates throughout the country supported inter herd transmission through local animal trade (4). The fact that there were no fatal cases in Poland previously described as associated with BCoV could have several explanations. Primarily, while it has been reported that similarly to other countries, respiratory and enteric diseases are the most common causes of calf mortality in Poland, screening to identify potential infectious factors involved is not routinely performed (27). Additionally while association between BCoV infection and respiratory signs was described, the severity of the disease may depend on other factors such as host susceptibility or environmental conditions (18, 19). In the case described in our study, the most probable initiating factor was stress associated with the movement of the animals, as the first symptoms appeared shortly after the introduction of the animals to the herd. The short, only one day gap between introduction of animals and onset of the disease additionally proves that animals were already infected at the time they arrived in the herd as previous studies showed that BCoV incubation time is at least two days (13). Transport of cattle, and the stress associated with it, are well recognized risk factors for BRDC. It was shown that inflammation caused by stress may weaken anti-viral immunity of the host (9). Reduced immunity, coupled with high density of animals during transport, facilitates spread of the viral infections and occurrence of disease (11). This was described in cattle transported from France to Italy where increased prevalence of infections with

BRSV and BCoV was observed shortly after arrival at the destination (7). Increased shedding of BCoV shortly after arrival at a new feedlot was also described in USA, and Canada although it was not always accompanied by an increase in symptoms of respiratory disease (21, 22, 26). Previously is was shown that the longer the transport of the cattle lasts the BRD morbidity increases, while in case of our study infected cattle originated from within Poland so the distance between farms was relatively short (17). Nevertheless, even local transport could affect the health of the animals as stressors like handling during loading/unloading or conditions during transport (ventilation, density of animals, commingling etc) are independent on the distance (6). In case of our study signs of BRDC were observed solely in newly introduced animals. However it can't be excluded that infection was also spread to other animals in the herd that were less susceptible to disease.

In our study both respiratory and enteric symptoms were observed in parallel, which is not uncommon in case of BCoV infections as it is known that depending on environmental circumstances and host factors, the same strain of this virus could be associated with different forms of disease (10, 13). The factor that could play a role in a severe course of infection might be co-infection with other, non-viral pathogens (25). However, a study performed in 2020 in Italy, showed that severe cases of respiratory and enteric disease accompanied by fatalities, could also be caused solely as a result of BCoV infections, with no other viral or bacterial infection agents involved (2). The case described in our study was even more drastic, as the majority of animals exhibiting signs of infection died (8 of 11 – 72.7%) whereas in the Italian study the mortality rate was only 20% (2). Our study is therefore a serious warning that BCoV infections should not be overlooked, and prevention and routine diagnostics for this pathogen should be considered in the future, especially in calves, as they represent an age group more susceptible to infection (19). It also indicates that the health status of calves newly introduced into a herd should be monitored, and that they should undergo quarantine.

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