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DETECTION AND QUANTITATIVE ASSESSMENT OF VIRAL AND BACTERIAL PATHOGENS IN BOVINE RESPIRATORY DISEASES BY REAL-TIME-qPCR

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Abstract

Bovine respiratory diseases are widespread in all countries with intensive animal husbandry and cause significant economic damage. They are the result of a synergistic interaction of several viruses and bacteria, predominantly of the *Pasteurellaceae* family. Clinical signs and pathological changes in internal organs depend on the presence or absence of a particular pathogen. Mass outbreaks occur when animals from different sources are mixed. The etiological structure of such outbreaks has been sufficiently studied, however, there is insufficient data on the distribution of bacteria and viruses in the respiratory tract and their quantitative determination. The article presents the results of studying the etiological structure of the outbreak of respiratory diseases in the big dairy farm after the import of cattle, during which more than 400 animals of different age and sex groups died. Samples of internal organs of 58 dead animals of different ages were examined. When studying the etiological structure of the outbreak, standard bacteriological methods were used, viral agents were identified by PCR by gel electrophoresis, and real-time PCR was used to quantify all detected infectious agents. In total, 9 viruses and bacteria were identified, of which the respiratory syncytial virus of cattle (BRSV, Bovine Respiratory Syncytial Virus, genus *Pneumovirus*, family *Paramyxoviridae*) and bacteria of the *Pasteurellaceae* family played a leading etiological role. Using quantitative PCR, the concentrations of the virus and bacteria *Pasteurella multocida* and *Mannheimia haemolytica* were determined in the respiratory tract organs of 13 calves of different ages with similar clinical signs, pathological changes and the presence of three pathogens in the respiratory tract organs. The concentration of agents ranged from 0.1±0.03 to 4.8±0.47 $\log_{10}$ genomic equivalents (GE)/ml for BRSV, from 1.3±0.60 to 4.1±0.30 $\log_{10}$ GE/ml for *P. multocida*, and from 1.9±0.03 to 4.9±0.67 $\log_{10}$ GE/ml for *M. haemolytica*. The concentration and distribution of pathogens in the organs of calves of different ages differed. BRSV was detected in a wider range of respiratory organs, both free from bacteria and colonized by them. In the lungs, the concentration of the virus was higher than in the tracheal and bronchial exudate. *P. multocida* was present only in the upper and middle lobes of the lungs of 2.5-4-month-old calves at approximately equal concentrations in acute bronchopneumonia. The degree of colonization of the lungs by this bacterium increased with age and in calves at the age of 6 months its number reached maximum values in the upper and middle lobes of the lungs, pulmonary lymph nodes and washes from the mucous membranes in chronic bronchopneumonia. *M. haemolytica* was detected in acute bronchopneumonia in calves at the age of 2.5 months in a minimum amount in the middle lobes of the lungs, in a maximum amount in tracheal and bronchial exudates. The results showed that the virus and bacteria multiply in different parts of the lungs without suppressing each other, which confirms the effect of their synergistic interaction and leads to an increase in the severity of the course of pneumonia. Quantification of viruses and bacteria by real-time PCR can be a useful tool for studying the pathogenesis of mixed viral-bacterial infections in vivo. The results obtained underline the role of the BRSV in the development of pulmonary pasteurellosis.

Keywords: cattle, respiratory infections, real-time PCR, quantitative analysis, respiratory syncytial virus, *Pasteurella multocida*, *Mannheimia haemolytica*, synergism

Bovine respiratory diseases are widespread in all countries with intensive

animal husbandry and cause significant economic damage [1, 2]. They are usually a result of synergistic interaction of several viruses and bacteria, predominantly of the *Pasteurellaceae* family. Viral pathogens include the infectious bovine rhinotracheitis (IBR) virus (IBRV, BoHV-1 or bovine herpes virus-1, synonymous with the Bovine alphaherpesvirus 1, genus *Varicellovirus*, the *Herpesviridae* family), bovine viral diarrhea virus types 1-3 (BVDV1-3, synonymous with Pestivirus A, B, and H, genus *Pestivirus*, the *Flaviviridae* family), bovine parainfluenza type 3 (BPIV-3 synonymous with bovine respirovirus 3, genus *Respirovirus*, the *Paramyxoviridae* family), and bovine coronavirus (BCoV, genus *Betacoronavirus*, the *Coronaviridae* family), and a few more [3, 4]. In practice, various viral-bacterial associations may occur, where one pathogen is dominant or absent; this affects the severity of the disease, as well as the nature of pathological changes [5]. Often, such outbreaks happen when a farm receives mixed livestock in bulk, including imported livestock.

Bovine respiratory syncytial virus (BRSV, genus *Orthopneumovirus*, the *Pneumoviridae* family) is one of the etiological agents of the bovine respiratory disease complex [6-9]. Animals of all breeds and ages are susceptible [10]. The virus may cause bronchitis, interstitial pneumonia, and pulmonary emphysema on its own; however, its key danger lies in the ability to create a predisposition to bacterial pneumonia [11, 12]. The papers [12-16] describe the synergy of the virus with the bacteria *Pasteurella multocida*, *Mannheimia haemolytica*, *Histophilus somni*.

Literature reports no data on BRSV accumulation in different compartments of the respiratory tract in naturally infected animals, nor any data on co-infection with *P. multocida*, *M. haemolytica*. Such studies could help significantly expand the knowledge of how BRSV contributes to the occurrence and progression of bovine viral-bacterial infections. The virus could be not just a trigger but also an independent pathogen replicating in organs and tissues in bacterial presence.

The novelty of the obtained results consists in collecting new data on the detection and finding the concentration of BRSV, *P. multocida*, and *M. haemolytica* genomes in animal respiratory tracts should a mass outbreak of respiratory diseases occur at a large dairy farm that has recently imported animals from abroad.

The goal was to study the etiological structure of a mass outbreak of bovine respiratory infection in a limited population and to find the concentration of BRSV genomes, *P. multocida,* and *M. haemolytica* in bovine respiratory tracts using real-time quantitative PCR.

Materials and methods. For testing, the researchers sampled nasal discharge, tracheal and bronchial exudate, mucous membranes of the trachea and large bronchi, and pulmonary lymph nodes, as well as various parts of the lungs from 58 differently aged Holstein animals that had succumbed on Day 7 to Day 14 after the first clinical signs. Samples were collected within 2 hours of death, frozen immediately, then delivered to the laboratory within 12 hours. Four hundred sixty-four samples were made in total. The farm's livestock was subject to 2-year morbidity monitoring.

The sampling technique was detailed earlier in [17, 18]. The samples were immediately frozen and then delivered to the laboratory, where they were split into two parts. The first part would be immediately tested for infectious agents; the second part was stored at –80 °C to further find the concentrations of pathogens.

Upon thawing, homogenized samples were centrifuged at 10,000 rpm for 5 min; 100 μl of clarified supernatant was used to isolate RNA and DNA as described in [17, 18]. Step 1 of studying the etiological structure of the outbreak

consisted in identifying the viral agents in PCR by gel electrophoresis using primer pairs and PCR parameters published earlier for BOHV-1 [17], BVDV-1 and BVDV-2 [18], BRSV [19], BPIV-3 [20], BCoV [21].

Bacteriological studies followed the World Organization for Animal Health (OIE) Guidelines [22]. Bacteria were isolated on artificial agars: blood meat peptone agar (BMPA) and Hottinger agar based on meat digest (10% horse serum added); they were typed in PCR using primers as described in [23-25].

Reverse transcription used the Reverta-L kit (Rospotrebnadzor CRIE, Russia) per the manufacturer's manual.

To quantify BRSV, *P. multocida,* and *M. haemolytica*, the researchers ran RT-PCR on 104 biosamples stored at –80 °C; preliminary studies identified only these three pathogens simultaneously.

Quantitative RT-PCR (*RT-qPCR)* used 30 μl of the mixture containing 5 μl of cDNA or DNA, 0.1 μM of each primer and probe as recommended for the tested pathogens [26–28], and a ready-made reagent mix: BioMaster PCR-RV (Biolabmix, Russia). The amplification program was as follows: 5 min at 95 °C, followed by 45 cycles: 15 s at 95 °C, 1 min at 60 °C. All reactions were run at a Real-time CFX96 Touch amplifier (Bio-Rad, USA).

Positive control samples (PCSs) were obtained using the TA Cloning® Kit with pCR™2.1 vector (In-vitrogen, USA) by cloning fragments of genes (*N* for BRSV, bovine *gapdh*, *kmt1 for P. multocida*, and *sodA for M. haemolytica*) into the pCR™ 2.1 cloning vector. PCS concentration was determined using the Quant-iT™ dsDNA Assay Kit, HS (Invitrogen, USA) and a QUBIT 4 fluorimeter (Invitrogen, USA), then converted into copy counts (see http://molbiol.ru/scripts/01_07.html for the converter software). Five milliliters of the PCS was added per reaction. Gene fragments (PCR specificity controls) were sourced from the strains RSB (BRSV), 1231 *(P. multocida)*, and S1-16 *(M. haemolytica)* obtained from the microorganism collection of the All-Russian Research Institute of Experimental Veterinary Medicine. The same strains were used to evaluate the sensitivity and reproducibility of reactions, and to calculate the coefficient of determination R^2.

To quantify pathogen RNA and DNA in the sample, a standard amplification curve was plotted, PCSs of known concentration were diluted 10-fold. The quantities of BRSV RNA and bacterial RNA in samples were estimated by comparing the cycle threshold of the sample against the standard curve; the quantities were then written in $\log_{10}$ genomic equivalents (GE for viral RNA or bacterial DNA) per 1 ml of the suspension (N $\log_{10}$ GE/ml), then normalized in relation to 10^5 GE of bovine *gapdh* as described in [29].

RT-qPCR sensitivity was tested in three repetitions.

The results were processed statistically in Statistica 8 (StatSoft, Inc., USA). For processing, the researchers calculated the means (*M*) and the standard errors of the mean (±SEM).

Results. The research was carried out at a dairy farm in possession of 1080 Holstein cows producing 7000.00 liters of milk per year on average; it was initiated after the importation of livestock from abroad that caused a respiratory disease outbreak in animals of all ages. As of the time of the study, the animals were not being vaccinated against viral diseases. The animals were stabled all-year-round pursuant to physiological and zootechnical standards.

In 2016, the farm imported 752 high-productivity heifers. These were housed separately from the local livestock; however, strict quarantine was not observed; 40 (5.3%) of the imported animals showed clinical signs of a disease, of which 10 (1.3%) soon succumbed to acute fibrinous pneumonia. Over the course

of 2017, these heifers produced 700 calves, 160 (22.8%) of which died before 30 days of age and had the same diagnosis. One month later, clinical signs of the disease were found in local animals including calves aged 1 to 6 months. Of the 600 calves, 490 (81.6%) contracted the disease, 240 (48.9%) succumbed to it. Beside the calves, the disease was found in 400 local cows; emergency slaughter and death count totaled 30 heads or 7.5%. Calves aged 10 days to 1 month were less susceptible. Clinical examinations revealed suppression, refusal of feed, fever, rapid abdominal breathing (mouth open, tongue out), lowered neck and head, abundant foaming at the mouth. Local calves were diagnosed with acute catarrhal-fibrinous bronchopneumonia postmortem. Their lungs showed lobar pneumonia and affected anteroventral sites: red hepatization in calves aged 2.5 months or younger, gray hepatization in older calves. Interlobular septa were edematous and impregnated with gelatinous exudate; they contained fibrin. Incisions showed lung tissue to be hemorrhagically inflamed and compacted with sites of necrosis. Pulmonary emphysema and edema were reported in some cases.

1. Primer pairs and PCR parameters used to identify viruses in biosamples of fallen Holstein animals of various ages after a mass outbreak of respiratory diseases (Novosibirsk Province, 2017)

Pathogen	Gene	Sequence (5′→3′)	Amplification protocol	DNA fragment size, bp	Reference
BoHV-1	*gB*	ACGTGCTGCTCAACGTGTAC AGGACGAGCTCGCGGATATA	95 °C 5 min; 95 °C 1 min, 54 °C 1 min, 72 °C 1,5 min (35 cycles); 72 °C 5 min	464	[17]
BVDV-1 BVDV-2	*NS5B*	GAGATCTTTCACACAATAGCTG GAACCTAAGAACTAAATCGG TGTTTCACCCAGTTATACATGC	95 °C 5 min; 95 °C 45 c, 60 °C 45 c, 72 °C 1 min (35 cycles); 72 °C 5 min	356 586	[18]
BRSV	*gF*	CATCAATCCAAAGCACCACACTGTC GCTAGTTCTGTGGTGGATTGTTGTC	95 °C 5 min; 95 °C 30 c, 60 °C 30 c, 72 °C 45 c (35 cycles); 72 °C 5 min	371	[19]
BPIV-3	*M* gene	GATCAGGAACTCTTAAAGGC TTTTCCCGACCCCTTCTAT	95 °C 5 min; 95 °C 15 c, 57 °C 25 c, 72 °C 30 c (40 cycles); 72 °C 5 min	739	[20]
BCoV	*N* gene	GCCGATCAGTCCGACCAATC AGAATGTCAGCCGGGGTAT	95 °C 5 min; 95 °C 30 c, 55 °C 30 c, 72 °C 45 c (35 cycles); 72 °C 5 min	407	[21]

Step 1 of studying the etiological structure of the outbreak consisted in identifying the viral agents in PCR with separation by gel electrophoresis using primer pairs and PCR parameters shown in [18-21, 23-25], see Tables 1 and 2.

2. Primer pairs and PCR parameters used to type the bacteria isolated on artificial nutrient agar media from biosamples of fallen Holstein animals of various ages after a mass outbreak of respiratory diseases (Novosibirsk Province, 2017)

Pathogen	Gene	Sequence (5′→3′)	Amplification protocol	DNA fragment size, bp	Reference
Mannheimia haemolytica	*sodA*	GACTACTCGTGTTGGTTCAGGCT CGGATAGCCTGAAACGCCT	95 °C 5 min; 95 °C 15 c, 57 °C 20 c, 72 °C 30 c (45 cycles); 72 °C 5 min	126	[23]
Pasteurella multocida	*kmt1*	ATAAGAAATAACTCAACATGGAAAT GAGTGGGCTTGTCGGTAGTCTT		211	[23]
Mycobacterium bovis	*urvC*	TTACGCAAGAGAATGCTTCA TAGGAAAGCACCCTATTGAT	95 °C 5 min; 95 °C 15 c, 52 °C 20 c, 72 °C 30 c (45 cycles); 72 °C 5 min	1600	[24]
Histophilus somni	*16S rDNA*	GAAGGCGATTAGTTTAAGAG TTCGGGCACCAAGTRTTCA	94 °C 5 min, 95 °C 1 min, 55 °C 1 мин, 72 °C 1 min (35 cycles); 72 °C 5 min	397	[25]

Bacteriological studies isolated and typed *M. haemolytica* (53.4%) and *P. multocida* (60.3%) in most animals, with *H. somni* (12.3%) and *Mycobacterium bovis* (6.9%) being rarer pathogens. Five viruses were found in the organs of dead

animals, mostly BRSV (65.5%), sometimes BCoV (22.4%) and BPIV-3 (20.7%). BoHV-1 and BVDV-1 were rarely detected: 6.9% and 8.6%, respectively. Twenty-seven animals had BRSV and BCoV: monovariant in four calves and in two- or three-virus association in 23 specimens, see Table 3.

3. Detection rates (number/%) **of respiratory pathogens in biosamples of fallen Holstein animals of various ages after a mass outbreak of respiratory diseases** (Novosibirsk Province, 2017)

Age	*n*	BRSV	BHV1	BVDV1	BVDV2	BPIV	BCoV	*Mannheimia haemolytica*	*Pasteurella multocida*	*Histophilus somni*	*Mycobacterium bovis*
Calves aged 30 days or younger	16	11/68.8	1/6.3	5/12.5	0	1/6.3	7/31.2	3/18.75	12/75.0	0	1/6.25
Calves aged 2-3 months	10	10/100.0	0	0	0	1/10.0	4/40.0	7/70.0	7/70.0	2/20.0	0
Calves aged 4-6 months	12	12/100.0	0	0	0	2/16.7	2/16.7	9/75.0	8/66.7	1/8.3	0
Heifers	10	2/20.0	3/30.0	0	0	5/50.0	0	5/50.0	3/30.0	3/30.0	1/10.0
Cows	10	3/30.0	1/10.0	0	0	3/30.0	0	7/70.0	5/50.0	2/20.0	2/20.0
Total	58	38/65.5	4/6.9	5/8.6	0	12/20.7	13/22.4	31/53.4	35/60.3	8/13.8	4/6.9

Pathogen detection rates were age-dependent. Calves aged 30 days or younger were more likely to have *M. haemolytica* (75.0%) and BRSV (68.8%), less likely to have *P. multocida* (18.8%) and BCoV (18.8%). Of calves aged 2 to 3 months, 100% had BRSV, 70% had *P. multocida,* 70% had *M. haemolytica,* 30% had BPIV-3. Of calves aged 4 to 6 months, 100% had BRSV, 75% had *M. haemolytica*, and 65.7% had *P. multocida*. BCoV and BPIV-3 were found in 25.0% and 16.7% of the specimens, respectively. Cows and heifers were 20% more likely to have *M. haemolytica* than *P. multocida*. BRSV in them was found in 20% and 30% of specimens in each group, respectively.

Three pathogens (BRSV, *M. haemolytica*, and *P. multocida*) were most prevalent in organ samples from nearly all respiratory tract compartments. For this reason, qPCR was then used to find the concentrations of these agents in various compartments of the respiratory tract where all the three pathogens had been found. Table 4 shows nucleotide sequences of the primers and probes used to that end.

4. Primer pairs and probes used in RT-qPCR to quantify the concentrations of major infectious agents in biosamples of fallen Holstein animals of various ages after a mass outbreak of respiratory diseases (Novosibirsk Province, 2017)

Target	Gene	Sequence (5′→3′)	Reference
BRSV	*N*	GCAATGCTGCAGGACTAGGTATAAT ACACTGTAATTGATGACCCCATTCT (FAM)ACCAAGACTTGTATGATGCTGCCAAAGCA(BHQ1)	[26]
Bos taurus	*gapdh*	GATGGTGAAGGTCGGAGTGAAC GTCATTGATGGCGACGATGT (ROX)CTGGTCACCAGGGCTGCTT(BHQ2)	[27]
Pasteurella multocida	*kmt1*	ATAAGAAACGTAACTCAACATGGAAATA GAGTGGGCTTGTCGGTAGTCTT (FAM)AAACCGGCAAATAACAATAAGCTGA(BHQ1)	[28]
Mannheimia haemolytica	*sodA*	GACTACTCGTGTTGGTTCAGGCT CGGATAGCCTGAAACGCCT (ROX)CTGGTTAGCGGTTGAAACAACGG(BHQ2)	[28]

RT-qPCR had a detection limit of 12 to 18 GEs per reaction or 1.08 $TCID_{50}$ for BRSV, 0.15 CFU for *M. haemolytica* and *P. multocida*, the coefficient of determination (R^2) ranging from 0.95 to 0.99, see Table 5.

5. Reproducibility and sensitivity of RT-qPCR test for quantification of major infectious agents in biosamples of fallen Holstein animals of various ages after a mass outbreak of respiratory diseases (Novosibirsk Province, 2017)

Pathogen	Sample	Coefficient of determination R^2	Minimum detectable concentration	GE to $TCID_{50}$ or GE to CFU ratios
BRSV	PC	0.98	18 GE	16.6
	Strain RSB	0.95	1.08 $TCID_{50}$	
Mannheimia haemolytica	PC	0.99	12 GE	80.0
	Strain S1-16	0.97	0.15 CFU	
Pasteurella multocida	PC	0.99	13 GE	86.7
	Strain 1231	0.96	0.15 CFU	

N o t e. PC stands for positive control samples, GE stands for genomic equivalents.

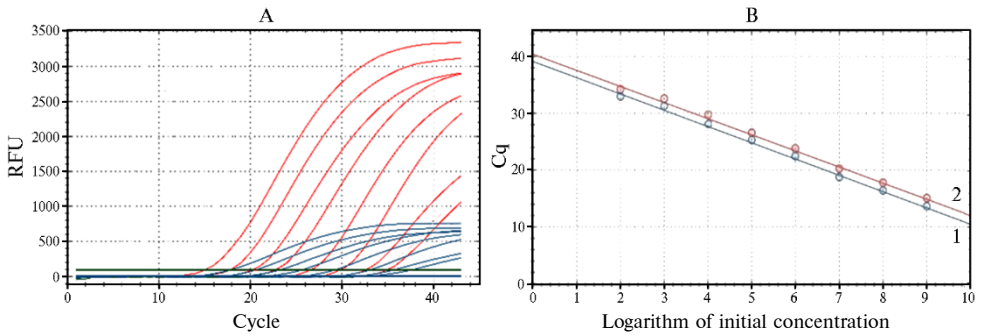

Fig. 1. Multiplex PCR curve (A) **and dynamic range of multiplex PCR readings (standard curve)** (B) **for detection of the genes *N* of BRSV and *gapdh* of *Bos taurus* in biosamples of fallen Holstein animals of various ages after a mass outbreak of respiratory diseases** (Novosibirsk Province, 2017). A: The X-axis shows PCR cycles, the Y-axis shows fluorescence in relative fluorescence units (RFU). B: The X-axis shows the sample quantity, N $\log_{10}$ GE/ml, the Y-axis shows the threshold cycle Cq (○ standard, × unknown, 1: FAM E = 123.4%, R^2 = 0.993, slope = –2.865, y-int = 39.133, 2: ROX E = 125.3%, R^2 = 0.995, slope = –2.835, y-int = 40.377). Blue lines are for the FAM probe (BRSV *N* gene); red lines are for the ROX probe (*Bos taurus gapdh* gene).

Fig. 1 shows the curve of multiplex PCR for the detection of BRSV *N* and *Bos taurus gapdh* genes, as well as the dynamic range of readings; Fig. 2 shows the same metrics for multiplex PCR detecting *P. multocida kmt1* and *M. haemolytica sodA* genes.

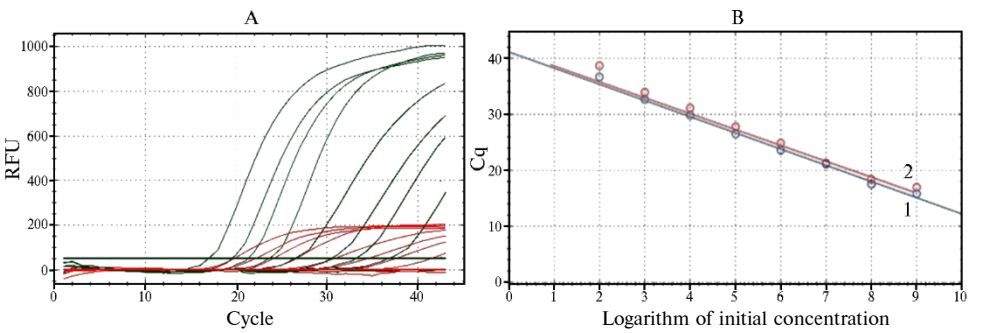

Fig. 2. Multiplex PCR curve (A) **and dynamic range of multiplex PCR readings (standard curve)** (B) **for detection of the genes *kmt1 Pasteurella multocida* and *sodA Mannheimia haemolytica* in biosamples of fallen Holstein animals of various ages after a mass outbreak of respiratory diseases** (Novosibirsk Province, 2017). A: The X-axis shows PCR cycles, the Y-axis shows fluorescence in relative fluorescence units (RFU). B: The X-axis shows the sample quantity, N $\log_{10}$ GE/ml, the Y-axis shows the threshold cycle Cq (○ standard, × unknown, 1: FAM E = 121.3%, R^2 = 0.958, slope = –2.,899, y-int = 41.182, 2: ROX E = 125.0%, R^2 = 0. 993, slope = –2.839, y-int = 41.488). Blue lines are for the FAM probe (gene *kmt1 P. multocida*); red lines are for the ROX probe (*sodA M. haemolytica*).

Table 6 shows the results of quantifying BRSV RNA and P. mul*tocida and M. haemolytica* DNA in biomaterial sampled from dead animals.

6. Concentration (log_{10} GE/ml suspension) of **Bovine Respiratory Syncytial Virus, *Pasteurella multocida*, *Mannheimia haemolytica* in biosamples of fallen Holstein heifers of various ages after a mass outbreak of respiratory diseases at a large livestock farm upon importation of animals** (Novosibirsk Province, 2017)

Biomaterial	Age								
	2.5 months			4 months			6 months		
	BRSV	*P. multocida*	*M. haemolytica*	BRSV	*P. multocida*	*M. haemolytica*	BRSV	*P. multocida*	*M. haemolytica*
Lung:									
cranial lobe	–	3.1±0.22	4.1±0.67	3.3±0.50	3.2±0.60	3.3±0.46	–	3.4±0.31	4.4±0.46
middle lobe	4.8±0.47	1.9±0.39	1.9±0.03	2.9±0.27	2.5±0.09	2.4±0.46	1.5±0.21	3.1±0.32	2.3±0.12
caudal lobe	2.1±0.30	–	2.5±0.13	1.8±0.21	–	3.5±0.31	1.3±0.5	–	3.1±0.27
Bronchial lymph nodes	–	–	2.3±0.21	0.1±0.03	–	2.1±0.12	–	2.1±0.09	2.1±0.55
Nasopharyngeal swab	–	–	2.8±0.35	2.1±0.25	–	4.1±0.30	–	2.2±0.55	1.6±0.30
Bronchotracheal swab	–	–	4.9±0.67	2.5±1.30	–	3.3±0.55	0.3±0.21	1.3±0.60	2.1±0.58
Mucosa:									
tracheal	0.5±0.03	–	–	–	–	–	–	–	–
bronchial	1.3±0.21	–	2.3±0.39	1.2 ±0.29	–	–	0.3±0.21	–	–

N o t e. GE stands for genomic equivalents; «–» means undetectable concentrations of pathogens.

The Table 6 shows BRSV concentration to vary from 0.1±0.03 to 4.8±0.47 $\log_{10}$ GE/ml, *P. multocida* and *M. haemolytica* to vary from 1.3±0.60 to 4.1±0.30 $\log_{10}$ and from 1.9±0.03 to 4.9±0.67 $\log_{10}$ GE/ml, respectively, in calves of various ages. The lungs had higher viral concentrations than tracheal or bronchial exudate, a sign of predominant pulmonary tropism of the virus.

P. multocida was found only in upper and middle lobes in calves aged 2.5 months (3.1±0.22 and 1.9±0.39 $\log_{10}$ GE/ml, respectively) and 4 months (3.2±0.60 and 2.5±0.09 $\log_{10}$ GE/ml, respectively) in approximately equal concentrations. This bacterium's colonization of the lungs correlated positively with age, as 6-month calves had up to 3.4±0.31 and 3.1±0.32 $\log_{10}$ GE/ml in upper and middle lobes. Besides, calves of this age group had *P. multocida* in pulmonary lymph nodes and mucosal swabs. This fact confirms the dominant role *of P. multocida* in the etiology of chronic bronchopneumonia in older calves.

In calves aged 2.5 months, *M. haemolytica* had the lowest concentration (1.9±0.03 $\log_{10}$ GE/ml) in the middle lobes and the highest concentration (4.9±0.67 $\log_{10}$ GE/ml) in bronchial exudate.

Thus, the detection percentages show the RSV and *M. haemolytica*, *P. multocida* to have played a key etiological role in the post-importation outbreak at the dairy farm. These results are consistent with the international researchers' reports on the synergistic interaction of the virus with *Pasteurellaceae* in severe animal bronchopneumonia [1, 4, 14, 16].

The respiratory syncytial virus is believed to have pathogenetic effects only in early stages of infection, thus being undetectable (or rarely detectable) if the lungs are colonized by bacteria, whereby it disappears after a transit infection [3, 6-9]. The data reported herein suggests that BRSV is present in the lungs even when colonized by *P. multocida* and *M. haemolytica*.

Bacteriological diagnosis is a time-consuming procedure, and its effectiveness depends on many factors including the use of antibacterial drugs, compliance with biosampling guidelines, freezing and thawing effects, as well as the presence of other microorganism species in the respiratory organs [1].

Detection and quantification of various pathogens in naturally infected animals by real-time PCR can be a useful tool for studying the pathogenesis of mono or mixed infections in vivo. The researchers did not find any results of similar studies in the available literature. There are reports of quantifying the concentration of the virus in nasal discharge or bronchoalveolar lavage in calves infected experimentally with highly pathogenic virus strains; those, however, concern monoinfection only. No earlier reports were found on finding the virus concentration in naturally infected animals [30–33]. Some reports concern co-infection with BRSV and other respiratory viruses [34–35], BRSV and bacteria (*Histophilus somni* [13], *P. multocida*, *M. haemolytica*, and *Trueperella pyogenes*) as detected by multiplex PCR [36], as well as immunohistochemical detection of *P. multocida* and *M. haemolytica* infections [34]. However, these viral and bacterial agents had not been quantified before this study.

RT-PCR made it possible to identify and quantify the two bacteria in the respiratory tract organs of the tested calves; the quantities were low. It is not clear whether this could be attributable to the limitations of the method or to the weak reproduction of the bacteria in the respiratory tract within the specific timeframe.

The results reported herein show the RSV and the *Pasteurellaceae* bacteria to be well-capable of simultaneously, non-mutually-suppressed reproduction in various lung parts, which confirms their synergy [1, 4, 14, 16], as well as the *M. haemolytica* + *P. multocida* interaction, which makes the clinical signs and the course of pneumonia in animals more severe. Besides, compared to the bacteria, the virus was found in a broader range of biosamples (nasopharyngeal and bronchotracheal

swabs, tracheal and bronchial mucosa, bronchial lymph nodes, cranial, middle, and caudal lobes) taken from animals with signs of pulmonary pasteurellosis. The concentration of the BRSV genome was also determined in naturally infected animals in a broader range of respiratory organs than the researchers did on experimentally infected gnotobiotic calves in [30-33].

Thus, RT-PCR made it possible to evaluate the distribution of three pathogens in the respiratory tract organs in naturally infected calves and to quantify BRSV, *Pasteurella multocida,* and *Mannheimia haemolytica* concentrations, which varied from 0.1±0.03 to 4.8±0.47 $\log_{10}$ GE/ml, 1.3±0.60 to 4.1±0.30 $\log_{10}$ GE/ml, and 1.9±0.03 to 4.9±0.67 $\log_{10}$ GE/ml, respectively. BRSV had maximum concentrations in the lungs, a sign of its predominant pulmonary tropism. RT-PCR could be useful for studying the spread and reproduction dynamics of pathogens in mixed viral-bacterial infections, namely the pathogenesis and synergistic interaction of pathogens from different nosological groups in bovine respiratory diseases including pulmonary pasteurellosis. Besides, these findings highlight the importance of RT-PCR as a diagnostic method, as BRSV replicates weakly in cell cultures, and its infection is transit and short-lived.

REFERENCES

1. Brogden K.A., Guthmiller J.M. *Polymicrobial diseases.* Washington, 2002.
2. Gorden P.J., Plummer P. Control, management and prevention of bovine respiratory disease in dairy calves and cows. *Veterinary Clinics of North America: Food Animal Practice*, 2010, 26(2): 243-259 (doi: 10.1016/j.cvfa.2010.03.004).
3. Andrews A.H., Blowey R., Boyd H., Eddy R. Respiratory disease. In: *Bovine medicine: diseases and husbandry of cattle*. A.N. Andrews, R. Blowey, H. Boyd, R. Eddy (ed.). Blackwell Scientific Publications, Oxford, 2004, 1232.
4. Fulton R.W., Purdy C.W., Confer A.W., Saliki J.T., Loan R.W., Briggs R.E., Burge L.J. Bovine viral diarrhea viral infections in feeder calves with respiratory disease: interactions with *Pasteurella* spp., parainfluenza-3 virus, and bovine respiratory syncytial virus. *The Canadian Journal of veterinary Research*, 2000, 64(3): 151-159.
5. Ackermann M.R., Brogden K.A. Response of the ruminant respiratory tract to *Mannheimia (Pasteurella) haemolytica*. *Microbes and Infection*, 2000, 2(9): 1079-1088 (doi: 10.1016/s1286-4579(00)01262-4).
6. Brodersen B.W. Bovine respiratory syncytial virus. *Veterinary Clinics of North America: Food Animal Practice*, 2010, 26(2): 323-333 (doi: 10.1016/j.cvfa.2010.04.010).
7. Larsen L.E. Bovine Respiratory Syncytial Virus (BRSV): a review. *Acta Veterinaria Scandinavica*, 2000, 41(1): 1-24.
8. Sacco R.E., McGill J.L., Pillatzki A.E., Palmer M.V., Ackermann M.R. Respiratory syncytial virus infection in cattle. *Veterinary Pathology*, 2014, 51(2): 427-436 (doi: 10.1177/0300985813501341).
9. Valarcher J.R., Schelcher R., Bourhy H. Evolution of bovine respiratory syncytial virus. *Journal of Virology*, 2000, 74(22): 10714-10728 (doi: 10.1128/jvi.74.22.10714-10728.2000).
10. Murray G.M., More S.J., Clegg T.A., Earley B., O'Neill R.G., Johnston D., Gilmore J., Nosov M., McElroy M.C., Inzana T.J., Cassidy J.P. Risk factors associated with exposure to bovine respiratory disease pathogens during the peri-weaning period in dairy bull calves. *BMC Veterinary Research*, 2018, 14: 53 (doi: 10.1186/s12917-018-1372-9).
11. Fulton R.W., d'Offay J.M., Landis C., Miles D.G., Smith R.A., Saliki J.T., Ridpath J.F., Confer A.W., Neill J.D., Eberle R., Clement T.J., Chase C.C., Burge L.J., Payton M.E. Detection and characterization of viruses as field and vaccine strains in feedlot cattle with bovine respiratory disease. *Vaccine*, 2016, 34(30): 3478-3492 (doi: 10.1016/j.vaccine.2016.04.020).
12. Sudaryatma P.E., Nakamura K., Mekata H., Sekiguchi S., Kubo M., Kobayashi I., Subangkit M., Goto Y., Okabayashi T. Bovine respiratory syncytial virus infection enhances *Pasteurella multocida* adherence on respiratory epithelial cells. *Veterinary Microbiology*, 2018, 220: 33-38 (doi: 10.1016/j.vetmic.2018.04.031).
13. Agnes J.T., Zekarias B., Shao M., Anderson M.L., Gershwin L.J., Corbeil L.B. Bovine respiratory syncytial virus and *Histophilus somni* interaction at the alveolar barrier. *Infection and Immunity*, 2013, 81: 2592-2597 (doi: 10.1128/IAI.00108-13).
14. Singh K.J., Ritchey W., Confer A.W. *Mannheimia haemolytica:* bacterial-host interactions in bovine pneumonia. *Veterinary Pathology*, 2011, 48(2): 338-348 (doi: 10.1177/0300985810377182).
15. Tizioto P.C., Kim J., Seabury C.M., Schnabel R.D., Gershwin L.J., Van Eenennaam A.L., Toaff-Rosenstein R., Neibergs H.L., Taylor J.F. Immunological response to single pathogen challenge

with agents of the bovine respiratory disease complex: an RNA-Sequence analysis of the bronchial lymph node transcriptome. *PLoS ONE*, 2015, 10(6): e0131459 (doi: 10.1371/journal.pone.0131459).
16. Rice J.A., Carrasco-Medina L., Hodgins D.C., Shewen P.E. *Mannheimia haemolytica* and bovine respiratory disease. *Animal Health Research Reviews*, 2007, 8(2): 117-128 (doi: 10.1017/S1466252307001375).
17. Glotov A.G., Glotova T.I., Nekrasova N.V., Nefedchenko A.V., Goppe V.A. *Veterinariya*, 2005, 11: 20-23 (in Russ.).
18. Glotov A.G., Glotova T.I., Nefedchenko A.V., Grebennikova T.V., Alipper T.I. *Veterinariya*, 2007, 12: 27-29 (in Russ.).
19. Vilcek S., Elvander M., Ballagi-Pordany A., Bleak S. Development of nested PCR assays for detection of bovine respiratory syncytial virus in clinical samples. *Journal of Clinical Microbiology*, 1994, 32(9): 2225-2231 (doi: 10.1128/JCM.32.9.2225-2231.1994).
20. Horwood P.F., Gravel J.L., Mahony T.J. Identification of two distinct bovine parainfluenza virus type 3 genotypes. *Journal of General Virology*, 2008, 89(7): 1643-1648 (doi: 10.1099/vir.0.2008/000026-0).
21. Takiuchi E., Stipp D.T., Alfieri A.F., Alfieri A.A. Improved detection of bovine coronavirus *N* gene in faces of calves infected naturally by a semi-nested PCR assay and an internal control. *Journal of Virological Methods*, 2006, 13(2): 148-154 (doi: 10.1016/j.jviromet.2005.08.005).
22. OIE. *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 8th Edition*. Paris, France, 2018.
23. Nefedchenko A.V., Shikov A.N., Glotov A.G., Glotova T.I., Ternovoi V.A., Agafonov A.P., Sergeev A.N., Donchenko N.A. *Molekulyarnaya genetika, mikrobiologiya i virusologiya,* 2016, 34: 62-66 (doi: 10.18821/0208-0613-2016-34-2-62-66) (in Russ.).
24. Subramaniam S., Bergonier D., Poumarat F. Species identification of *Mycoplasma bovis* and *Mycoplasma agalactiae* based on the *urvC* genes by PCR. *Molecular and Cellular Probes*, 1998, 12(3): 161-169 (doi: 10.1006/mcpr.1998.0160).
25. Angen Ø., Ahrens P., Tegtmeier C. Development of a PCR test for identification of *Haemophilus somnus* in pure and mixed cultures. *Veterinary Microbiology*, 1998, 63(1): 39-48 (doi: 10.1016/S0378-1135(98)00222-3).
26. Boxus M., Letellier C., Kerkhofs P. Real time RT-PCR for the detection and quantitation of bovine respiratory syncytial virus. *Journal of Virological Methods*, 2005, 125(2): 125-130 (doi: 10.1016/j.jviromet.2005.01.008).
27. Zhao H., Liu J., Li Y., Yang C., Zhao S., Liu J., Liu A., Liu G., Yin H., Guan G., Luo J. Validation of reference genes for quantitative real-time PCR in bovine PBMCs transformed and non-transformed by *Theileria annulata*. *Korean Journal of Parasitology*, 2016, 54(1): 39-46 (doi: 10.3347/kjp.2016.54.1.39).
28. Nefedchenko A.V., SHikov A.N., Glotov A.G., Glotova T.I., Ternovoi V.A., Maksyutov R.A., Agafonov A.P., Sergeev A.N. Detection and genotyping *Pasteurella multocida* of five capsular groups in real time polymerase chain reaction. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2017, 52(2): 401-408 (doi: 10.15389/agrobiology.2017.2.401eng).
29. Jordan R., Shao M., Mackman R.L., Perron M., Cihlar T., Lewis S.A., Eisenberg E.J., Carey A., Strickley R.G., Chien J.W., Anderson M.L., McEligot H.A., Behrens N.E., Gershwin L.J. Antiviral efficacy of an RSV fusion inhibitor in a bovine model of RSV infection. *Antimicrobial Agents and Chemotherapy*, 2015, 59(8): 4889-4900 (doi: 10.1128/AAC.00487-15).
30. Antonis A.F.G. Age-dependent differences in the pathogenesis of bovine respiratory syncytial virus infections related to the development of natural immunocompetence. *Journal of General Virology*, 2010, 91(10): 2497-2506 (doi: 10.1099/vir.0.020842-0).
31. Blodörn K., Hägglund S., Gavier-Widen D., Eléouët J.F., Riffault S., Pringle J., Taylor G., Valarcher J.F. A bovine respiratory syncytial virus model with high clinical expression in calves with specific passive immunity. *BMC Veterinary Research*, 2015, 11: 76 (doi: 10.1186/s12917-015-0389-6).
32. Thomas L.H., Slott E.J., Collins A.P., Jebbett J. Experimental pneumonia in gnotobiotic calves produced by respiratory syncytial virus. *British Journal of Experimental Pathology*, 1984, 65: 19-28.
33. Tjørnehøj K., Uttenthal A., Viuff B., Larsen L.E., Røntved C., Rønsholt L. An experimental infection model for reproduction of calf pneumonia with bovine respiratory syncytial virus (BRSV) based on one combined exposure of calves. *Research in Veterinary Science*, 2003, 74(1): 55-65 (doi: 10.1016/s0034-5288(02)00154-6).
34. Yaman T., Büyükbayram H., Özyıldız Z., Terzi F., Uyar A., Keles Ö.F., Özsoy Ş.Y., Yener Z. Detection of bovine respiratory syncytial virus, *Pasteurella multocida*, and *Mannheimia haemolytica* by immunohistochemical method in naturally-infected cattle. *Journal of Veterinary Research*, 2018, 62(4): 439-445 (doi: 10.2478/jvetres-2018-0070).
35. Thonur L., Maley M., Gilray J., Crook T., Laming E., Turnbull D., Nath M., Willoughby K. One-step multiplex real time RT-PCR for the detection of bovine respiratory syncytial virus, bovine herpesvirus 1 and bovine parainfluenza virus 3. *BMC Veterinary Research*, 2012, 8: 37 (doi: 10.1186/1746-6148-8-37).
36. Zhang W., Liu X., Liu M., Ma B., Xu L., Wang J. Development of a multiplex PCR for simultaneous detection of *Pasteurella multocida*, *Mannheimia haemolytica* and *Trueperella pyogenes*. *Acta Veterinaria Hungarica*, 2017, 65(3): 327-339 (doi: 10.1556/004.2017.032).