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Original Research Article

THE PREVALENCE OF TEN PATHOGENS DETECTED BY A REAL-TIME PCR METHOD IN NASAL SWAB SAMPLES COLLECTED FROM LIVE CATTLE WITH RESPIRATORY DISEASE

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Summary: Respiratory diseases often correspond to primary infections with different pathogens of cattle, causing heavy economic losses in young stock and breeding herds. Between 2012 and 2014, nasal swab samples were collected from twenty-eight herds from 133 affected live cattle that were clinically suffering from symptoms of respiratory disease, pyrexia, cough, serous nasal and lacrimal discharge, increased respiratory rate, and breath sounds. Individual swab samples were tested in the laboratory using three commercial and one in-house real-time PCR methods, to detect nucleic acids of a total of ten different respiratory pathogens. *Pasteurella multocida* (*P. multocida*) was detected in 58.65% of samples, *Mannheimia haemolytica* (*M. haemolytica*) in 15.04%, while *Mycoplasma bovis* (*M. bovis*) and *Histophilus somni* (*H. somni*) were positive in 9.77% of nasal swab samples. Among viral pathogens, the highest prevalence (40.60%) was observed for bovine respiratory syncytial virus (BRSV), followed by bovine coronavirus (BCV) 12.03%, bovine para-influenza 3 (PI-3) 3.01%, and bovine viral diarrhea virus (BVDV) with 1.50% of positive samples. The less frequently detected viral pathogens were bovine herpes virus type 1 (BHV-1) and bovine adenovirus (BAdV) with 0.75% positive samples each. The new implemented molecular methods can be an important diagnostic tool for laboratories and farmers to improve the therapy, control, and prevention of respiratory disease in cattle herds.

Key words: bovine respiratory disease; nasal swab samples; diagnostics; real-time PCR detection; cattle

Introduction

Bovine respiratory disease (BRD) is the major cause of serious respiratory tract infections worldwide, often leading to high morbidity and mortality rates in cattle. The disease is considered to be a multifactorial disorder, produced with either stress or reduced immunity, allowing several pathogens to emerge. It inflicts considerable mortality and financial losses mainly in calves in dairy and beef herds. Viral and bacterial pathogens together with mycoplasma and environmental risk factors are the most common cause of diseases, ranging from common colds to life-threatening pneumonia (1). A large number of both RNA and DNA viruses uses the respiratory tract to initiate host infection. Infection may be restricted to certain sections of the airway system such as the trachea, bronchi, or alveoli. For some viruses, the respiratory tract may merely serve as a primary entry site from where infection spreads to other organs or tissues. An important defense strategy is the mucociliary clearance system. While some epithelial cells are specialized to produce and release mucins, other cells are equipped with cilia

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that enable them to contribute to the transport of the mucus with pathogens out of the respiratory tract. The most important viral pathogens associated with BRD are bovine respiratory syncytial virus (BRSV), bovine parainfluenza virus 3 (PI-3), bovine herpesvirus 1 (BHV-1), bovine adenovirus (BAdV), bovine coronavirus (BCV) and bovine viral diarrhea virus (BVDV) (2, 3). While BHV-1 and BVDV have already been eradicated in some European countries, infections with BRSV and BCV are endemic in the cattle population globally (4, 5). The infection of cattle with BAdV usually results in disease of the gastrointestinal or respiratory tract. Different serotypes of BAdV are divided into two subgroups. BAdV type 1, 2, 3, 9 and 10 comprise Group A, BAdV type 4, 5, 6, 7 and 8 comprise Group B (6). Only a few publications on the simultaneous detection of more than three different pathogens in case of respiratory or enteric diseases are available (7, 8, 9). Several studies present antibodies against etiologic agents of BRD, but these only indirectly confirm the previous infections with specific viral and bacterial agents in animals or herds (4-5, 10-12). Newly developed molecular methods significantly improved the diagnosis of respiratory tract infections, providing a fast and cost-effective tool for different pathogens, to determine the prevalence of respiratory viruses, bacteria, and mycoplasma in clinically affected cattle (13). Few reports about the prevalence on the respiratory disease of cattle in Slovenia exist, mainly obtained several years ago with conventional methods of bacteria or virus isolation (14–18). However, the traditional farming system in Slovenia with small isolated cattle herds is disappearing; herds are enlarging gradually and, in several cases, animals of different herds and ages are kept in a pen. In the new rearing system, young calves at the age of 1-3 weeks originating from several herds, are transported to beef units and grouping together, frequently with a combination of imported beef cattle. Sometimes, vaccines against respiratory disease are used, but almost no data about effectiveness is available. Antimicrobials are generally not used for disease prevention; sick animals are mostly treated individually with antibiotics.

The aim of this study was to determine the prevalence of ten respiratory pathogens detected by new implemented real-time PCR methods using nasal swab samples collected from affected cattle with respiratory disease.

Materials and methods

Sampling was conducted mostly in winter and spring periods from 2012 to 2014. All swab samples were collected into sterile swabs (Sigma Virocult®, MW 951S, UK) and were immediately sent to a laboratory. Nasal swab samples were collected from 133 live affected animals, originating from twenty-eight different Slovenian cattle herds identified with bovine respiratory disease. Five of them are feedlot cattle herds, four are dairy herds, and nineteen are traditional, combined herds, with milk and meat production. All of the sampled animals had abnormal sound on auscultation of the respiratory tract and most had either one or several of the following symptoms: fever >39 °C, elevated respiratory rate (> 40/min), cough or nasal and/or lacrimal discharge. In the case of an acute outbreak on a farm, 1 to 18 samples were collected from the same herd, only from clinically affected animals. As a control group of the study, ten animals from three farms without clinical signs of respiratory disease in the previous two months were selected, and nasal swab samples were collected from healthy animals and screened for ten pathogens.

After arrival at the laboratory, samples were homogenized and stored in a freezer at < -15 °C until testing. Total nucleic acids were extracted from 140 µl of homogenate using a commercial kit for RNA extraction (QIAamp® Viral RNA Mini Kit (Qiagen, Germany)) according to the manufacturer's instructions. Individual swab samples were tested by one in-house and three commercial real-time PCR methods, detecting specific nucleic acids of a total of ten different respiratory pathogens, including detection of endogenous internal positive control (IPC) for controlling the efficiency of extraction and the absence of inhibitors in individual samples. Samples were tested on a 96-tube microplate. On each microplate, the positive controls for all tested pathogens were included. A commercial TaqMan® real-time PCR kit for the detection of seven major ruminant pathogens (LSI VetMAX™ Screening Pack – Ruminants Respiratory Pathogens, LSI, France) allows the simultaneous detection of the *M. bovis*, *H. somni*, *P. multocida*, *M. haemolytica*, BCV, BRSV, and PI-3. For the detection of BVDV and BHV-1, another two commercial real-time kits (Kit TaqVet® BVDV "Screening" and LSI VetMAX™ IBR gB, both produced by LSI, France) were used

according to the producer's instructions. For the detection of BAdV, an in-house protocol was implemented with previously designed primers detecting BAdV, serotypes 4-8 (19). Real-time PCR was performed using the forward primer BAV4-8F 5'-CRA GGG AAT AYY TGT CTG AAA ATC-3', the reverse primer BAV4-8R 5'-AAG GAT CTC TAA ATT TYT CTC CAA GA-3' and the probe FAM-TTC ATC WCT GCC ACW CAA AGC TTT TTT-BHQ-1 targeting the hexon gene of BAdV (9). The reaction was performed in a total volume of 15 µl, using QuanTitec® Virus Kit (Qiagen, Germany) as follows: 8 µl of nuclease free water, 3 µl of 5x PCR Master Mix, 0,5 µl of the stock solution with 20 µM of BAV4-8F primer, 0,5 µl of the 20 µM of BAV4-8R primer, 0,5 µl of the stock solution with 10 µM of probe and 2,5 µl of the RNA/DNA template. The real-time PCR running program for BAdV was 95 °C for 15 min; followed by 45 cycles of 95 °C for 10 s, 54 °C for 30 s and 60 °C for 30 s. All real-time cyclings were performed on an Mx3005P thermocycler (Stratagene, USA) using protocol according to the manufacturer's instructions for commercial kits and the above-described protocol for BAdV detection. The fluorescent signal was detected after each annealing, and the results were presented as a cycle threshold value for individual samples. Analysis of real-time amplification curves was performed using commercial thermal cycler system software, and an "auto baseline" was used to determine fluorescence baselines.

Results

A total of 133 swab samples from live cattle with symptoms of respiratory disease and 10 swab samples from healthy cattle (control group) were successfully screened for 10 pathogens with the real-time PCRs method. In cattle with respiratory disease, *P. multocida* was detected in 78/133 (58.65%) of samples, *M. haemolytica* in 20/133 (15.04%), while *M. bovis* and *H. somni* were positive in 13/133 (9.77%) of nasal swab samples. The highest prevalence of viral pathogens was observed for BRSV 54/133 (40.60%), following BCV 16/133 (12.03%), PI-3 with 4/133 (3.01%) and BVDV with 2/133 (1.50%) of positive samples. The less frequently detected viral pathogens were BHV-1 and BAdV with 1/133 (0.75%) positive samples (Table 1).

At least one pathogen was detected in 110/133 (82.70%) of clinically affected cattle (Figure 1). In 43 samples (32.33%), only one pathogen was detected; *P. multocida* in 17 samples and BRSV in 16 samples, and *M. haemolytica* in five samples. The simultaneous detection of two different pathogens was observed in 46 samples (34.59%);

Table 1: The results of the detection of ten different pathogens in 133 nasal swabs samples, collected from live cattle suffering from respiratory disease together with a control group are presented. The ranges of cycle threshold values obtained by specific real-time PCR methods are presented for individual pathogens

Name of pathogen	Cattle with respiratory disease				Control group (healthy animals)		
	Number of tested samples	Number of positive samples	% of positive samples	Cycle threshold (Ct) range (mean)	Number of tested samples	Number of positive samples	Cycle threshold (Ct)
M. bovis	133	13	9.77%	20.88–37.81 (29.60)	10	0	-
H. somni	133	13	9.77%	26.63–41.97 (35.35)	10	1	35.35
P. multocida	133	78	58.64%	19.76–43.04 (30.41)	10	1	32.99
M. haemolytica	133	20	15.04%	27.12–43.54 (34.05)	10	0	-
BCV	133	16	12.03%	25.16–38.82 (31.61)	10	0	-
BRSV	133	54	40.60%	20.48–39.89 (29.53)	10	0	-
BPI-3	133	4	3.01%	20.81–42.05 (34.05)	10	0	-
BVDV	133	2	1.50%	28.73–35.34 (32,03)	10	0	-
BHV-1	133	1	0.75%	29.91 (29.91)	10	0	-
BAdV	133	1	0.75%	29.43 (29.43)	10	0	-

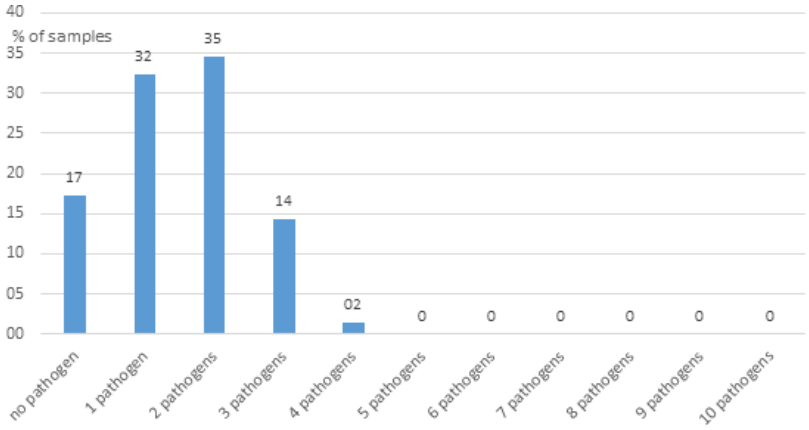

Figure 1: The percentage of negative and positive samples detected by real-time PCR method, with the presentation of the simultaneous detection of two to four different pathogens in nasal swab samples collected from live cattle suffering from respiratory disease

Table 2: The results of the detection of 10 different respiratory pathogens by real-time PCR methods in three different types of herds in which respiratory disease was observed

Type of herd	Number of nasal swab samples	Number of possible results	Number of positive samples	% of positive samples	Number of positive animals	% of positive animals
feedlot	29	290	67	23.1%	29	100.0%
combined	70	700	105	15.0%	61	87.1%
dairy	34	340	30	8.8%	20	58.8%

in 26 samples *P. multocida* and BRSV, in five samples *P. multocida* and *M. bovis*, while in four samples *P. multocida* with BCV or *P. multocida* with *H. somni* or *M. haemolytica* together with BCV. In 19 (14.29%) positive samples, the simultaneous detection of three pathogens was identified; in 13 samples, two bacteria and one virus were confirmed (Fig. 1). In two samples, four pathogens were detected, in one sample BHV-1, *M. bovis*, *H. somni* and *P. multocida*, while in one sample *M. haemolytica* with three different viruses (BRSV, BCV and PI-3) was detected. The high variability of cycle threshold (Ct) for different pathogens was observed from low to high viral loads in a sample; the summary results of these data are presented in Table 1 and for the individual samples in the supplementary file (Supplementary data). All 29 tested nasal swabs collected from feedlot herds were positive for at least one respiratory pathogen (100%), while samples from combined herds were positive for at least one respiratory pathogen in 87.1% and from dairy herds in 58.8% of the collected samples (Table 2). Up to five individually tested samples, which were collected from the same herd, showed a similar pattern of pathogens (data presented in Supplementary data). In the control group, out of ten healthy animals, only in one animal were *H. somni* and *P. multocida* detected with Ct values of 35.35 and 32.99, respectively.

Discussion

To our knowledge, this study represents the first report of the simultaneous detection of ten respiratory pathogens by using real-time PCR in cattle. According to the results of this study, *P. multocida,* BRSV, *M. haemolytica*, BCV, *H. somni* and *M. bovis* are common pathogens in Slovenian cattle herds with respiratory problems. BVDV is endemically present in about 30% of herds (20), while the occurrence of BHV-1 and PI-3 in Slovenia is low (16) and the previous observation was confirmed also in this study. A significant finding of our study was that the real-time PCRs approach used could detect the concurrent infection of five different viruses, four bacteria, and mycoplasma with positive results of at least one pathogen in 82.70% of nasal swabs samples. A high percentage (40.60%) of identified positive samples with BRSV confirmed that this virus is a significant problem of herds with respiratory infections,

frequently complicated with *P. multocida* and one or more other pathogens. In 67 samples, at least two respiratory pathogens were detected; in 39 (58.21%), *P. multocida* together with BRSV. Where BRSV resides in a population of cattle for the virus to survive is not well understood, but low biosecurity measures on farms may be the main reason for the high prevalence. Persistently infected calves may exist, and possible triggering mechanisms such as a change in temperature may induce shedding (21). The second most prevalent virus was BCV with 12.03%, confirming the regular circulation of this virus in the herds. In 2011 and 2013, the antibody status in 70 conventional dairy herds in Sweden ranged from 73.4 to 82.3% for BRSV and from 76.8% to 85.3% for BCV (10). According to the observation in our study, similar antibody prevalence could also probably be detected in Slovenia. Apparently, there are good chances for a herd to be free of these infections if good biosecurity is practiced, and the virus is not reintroduced, as was shown in a Norwegian survey (11). However, a significant proportion of Slovenian herds have low biosecurity standards with the introduction of animals from herds of unknown status. Imported calves can be the reason for the introduction of new respiratory pathogens and other infections. The circulation of very similar strains of BCV in Slovenia was confirmed with the sequencing of 21 positive samples collected between 2012 and 2013 with 99-100% nucleotide identity in RNA polymerase gene (17). Under the experimental condition, BRSV was detected in nasal secretions between Day 2 and Day 14, but the PCR methods may detect virus shedding for a longer period after infection than virus isolation, possibly due to neutralization by mucosal antibodies (22, 23). Although BVDV can be detected in persistently infected cattle or during acute infection of seronegative immunocompetent cattle, the results of our study with the detection of 1.5% of positive samples confirmed that this virus is only occasionally detected in cattle with respiratory disease and is not closely linked to occurrence of BRD, which is consistent with previous observation in Finland (12). The low percent of positive samples of PI-3 was also previously observed (12), confirming that PI-3 is not a significant causal factor in BRD. To our knowledge, the occurrence of BAdV 4-8 has not been studied earlier in Slovenia. The low detected prevalence of BAdV 4-8 is surprising in comparison to some previous observations and its global distribution (12), but also may be the result of the type of samples in this study and the limitation of used real-time PCR method, thus further research in Slovenia is needed, targeting all known BAdV (24). Previous observations that bacterial pathogens such as *M. haemolytica, P. multocida* or *M. bovis* may induce a response reaction in the epithelial cells that make them susceptible to virus infection was also confirmed in our study (25). Mycoplasmas are considered to be one of the causal factors of BRD, but often together with other pathogens. The detection of *M. bovis* in 9.77% of samples suggest that this pathogen might be significant in pathogenesis in feedlot cattle herds, especially in multiple pathogen infections. This observation is also supported with high pathogen loads in positive samples with Ct values between 20.88 and 32.32 for *M. bovis* in 11 out of 13 positive samples. Because no data regarding the prevalence of *M. bovis* is available for Slovenia, more research needs to be done in the future. In our study, at least one bacterial infection was detected in 92/133 (68.42%), frequently (64/133, 51%) in combination with one or more bacteria or viruses, which is consistent with previous observation (26). *P. multocida* is another common pathogen observed in 58.65% of affected animals in our study and, as was previously suggested, the antimicrobial treatments are necessary in all clinical cases of BRD, because all *Pasteurella* spp. isolates were susceptible to antibiotics (26). From our study, it was clearly shown that *H. somni* or *M. haemolytica* are also frequently present in affected animals, suggesting their importance for diagnosis of respiratory problems. In general, real-time PCR methods are more suitable for diagnostic applications than conventional methods of isolation. These techniques offer high sensitivity and provide specific results within a shorter period and for a larger number of pathogens in comparison to conventional methods, such as virus isolation or direct fluorescence antibody tests (8). Highly sensitive real-time PCR assays used in this study confirmed previous reports of naturally occurring viral and bacterial infection. Real-time RT-PCRs has also been used in disease pathogenesis studies to determine viral load and viral gene expression (23). From the obtained Ct values for individual pathogens and the comparison of Ct values of different pathogens, the laboratory interpretation of the results can provide valuable additional data to the on-field veterinarian regarding therapy. Na-

sal swab samples are suitable for the examination of single or several affected animals in a herd and can also be performed by the farmer. This has an advantage in comparison to a sampling of lung lavage or blood collection, which can be done only by a veterinarian and because these samples frequently have lower detection rates in comparison to swabs (12). As was previously observed, the integrity of samples can be affected, if swab samples are not stored at low temperature and immediately send to the laboratory (27); the cold chain strategy also has to be followed for respiratory disease samples. If three to five animals are tested from the same herd, the probability of obtaining at least one pathogen is increased, as can be concluded from this study. Molecular methods also provide the pooling of up to five samples from the same herd to reduce the costs of testing. The observation of this study confirmed that BRD is frequently the result of infection with different pathogens, and their fast laboratory identification could help veterinarians towards selecting the correct therapy for animals with BRD. Our results indicate that the nasal swabs sampling in combination with real-time PCR methods can be a useful tool for the rapid and cost-effective diagnosis and surveillance of viral and bacterial respiratory infection in cattle.

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UGOTAVLJANJE PRISOTNOSTI DESETIH PATOGENOV Z METODO PCR V REALNEM ČASU V ODVZETIH VZORCIH NOSNIH BRISOV PRI ŽIVEM GOVEDU Z ZNAKI RESPIRATORNEGA OBOLENJA

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Povzetek: Bolezni dihal so pri govedu pogosto posledica primarne okužbe z različnimi patogeni, ki pri teletih in tudi v plemenski čredi povzročijo veliko gospodarsko škodo. V letih od 2012 do 2014 smo v 28 govejih čredah odvzeli vzorce nosnih brisov pri obolelih živih živalih. Vzorčenje smo izvedli pri 133 živalih, ki so klinično kazale enega ali več znakov obolenja dihal, povišano telesno temperaturo, kašelj, serozni nosni in očesni izcedek, pospešeno dihanje in povišan zvok ob pregledu pljuč. Vzorce nosnih brisov smo testirali s tremi komercialnimi in eno novo uvedeno laboratorijsko metodo PCR v realnem času na prisotnost nukleinskih kislin desetih različnih patogenov. Prisotnost bakterije *Pasteurella multocida* smo ugotovili v 58.65 % vseh vzorcev, bakterije *Mannheimia haemolytica* v 15.04 %, pristonost bakterij *Mycoplasma bovis* in *Histophilus somni* pa smo ugotovili v 9.77 % vzorcev. Med iskanimi virusi smo bovini respiratorni sincicialni virus ugotovili v 40.60 % vzorcev, bovini koronavirus v 12.03 % vzorcev, virus parainfluence 3 v 3 %, virus bovine virusne diareje v 1.5 % vzorcev; najmanj pogosto smo ugotovili prisotnost bovinega herpesvirusa 1 in bovinega adenovirusa (0.75 % vseh vzorcev). Novo uporabljene molekularne metode predstavljajo pomembno diagnostično orodje za laboratorije in rejce in v goveji čredi pomagajo pri izbiri ustreznejše terapije, nadzoru in preprečevanju bolezni dihal.

Ključne besede: respiratorno oboljenje; vzorci nosnih brisov; diagnostika; PCR v realnem času; govedo