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Development of a one-run real-time PCR detection system for pathogens associated with bovine respiratory disease complex

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ABSTRACT. Bovine respiratory disease complex (BRDC) is frequently found in cattle worldwide. The etiology of BRDC is complicated by infections with multiple pathogens, making identification of the causal pathogen difficult. Here, we developed a detection system by applying TaqMan real-time PCR (Dembo respiratory-PCR) to screen a broad range of microbes associated with BRDC in a single run. We selected 16 bovine respiratory pathogens (bovine viral diarrhea virus, bovine coronavirus, bovine parainfluenza virus 3, bovine respiratory syncytial virus, influenza D virus, bovine rhinitis A virus, bovine rhinitis B virus, bovine herpesvirus 1, bovine adenovirus 3, bovine adenovirus 7, *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, *Trueperella pyogenes*, *Mycoplasma bovis* and *Ureaplasma diversum*) as detection targets and designed novel specific primer-probe sets for nine of them. The assay performance was assessed using standard curves from synthesized DNA. In addition, the sensitivity of the assay was evaluated by spiking solutions extracted from nasal swabs that were negative by Dembo respiratory-PCR for nucleic acids of pathogens or synthesized DNA. All primer-probe sets showed high sensitivity. In this study, a total of 40 nasal swab samples from cattle on six farms were tested by Dembo respiratory-PCR. Dembo respiratory-PCR can be applied as a screening system with wide detection targets.

KEY WORDS: bovine respiratory disease complex, diagnosis, taqMan real-time PCR

Bovine respiratory disease complex (BRDC) is one of the most prevalent cattle diseases, causing large economic losses in the cattle industry worldwide. For example, BRDC is estimated to account for 70–80% of all feedlot cattle morbidity and 40–50% of all cattle mortality, resulting in a loss of greater than US $500 million per year in the United States [16, 22]. In Japan, number of illness in the statistics mutual aid for livestock owners in 2014 reported by the Ministry of Agriculture, Forestry and Fisheries indicate that respiratory diseases accounted for 23% of illness in cattle (http://www.maff.go.jp/j/tokei/kouhyou/katiku_kyosai/) (in Japanese). Despite considerable research and attempts to manage hygiene to prevent BRDC, these measures have had a limited effect on reducing the impacts of this disease [29].

One of the difficulties in investigation and prevention of BRDC is its multi-factorial etiology, based on host interactions with multiple pathogens, such as virus and bacteria, including mycoplasma. The primary viral infection sometimes results in clinical symptoms, and it was exacerbated by secondary infections with bacterial pathogens [10]. Stressors, such as transportation, and host factors, such as nutritional status, raise the risk and severity of BRDC [17, 28]. Viruses, which are commonly associated with

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BRDC, include bovine viral diarrhea virus (BVDV), bovine coronavirus (BCoV), bovine parainfluenza virus 3 (BPIV-3), bovine respiratory syncytial virus (BRSV), bovine herpesvirus 1 (BHV-1), and bovine adenovirus 3 (BAdV 3) and 7 (BAdV 7) [12, 28]. In addition, a metagenomic approach to BRDC in cattle recently revealed that several other viruses may be involved in BRDC, such as influenza D virus (IDV), bovine rhinitis A virus (BRAV) and bovine rhinitis B virus (BRBV) [13, 23]. *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, *Trueperella pyogenes* and *Mycoplasma bovis* are bacteria consistently associated with BRDC [1, 11]. *Ureaplasma diversum* has also been isolated from BRDC cattle [8]. Infection with each single pathogen does not necessarily result in appearance of symptoms, but complex infections with a variety of pathogens, including the indigenous agents, develop severe conditions. Such multiple pathogens infection make it difficult to identify the etiology of BRDC rapidly.

To adopt appropriate measures, such as vaccination or hygiene management, and to minimize the economic loss of BRDC, it is necessary to quickly, accurately and comprehensively detect multiple pathogens present in varying proportions in each herd. Serological tests, pathogen isolation and PCR-based tests are currently available to diagnose BRDC in laboratories [7]. Most tests are based on a one assay-one pathogen approach, lacking comprehensiveness to use for rapid diagnosis. We previously developed a system to detect microbes in bovine diarrhea by TaqMan real-time PCR (Dembo diarrhea-PCR), permitting the simultaneous screening of 19 pathogens associated with diarrhea [31]. Advantages of TaqMan real-time PCR are high sensitivity, high specificity and simple operation. The objective of this study was to develop a system based on TaqMan real-time PCR that can detect 16 pathogens, including viruses and bacteria, associated with BRDC in one run (Dembo respiratory-PCR), similar to Dembo diarrhea-PCR.

MATERIALS AND METHODS

Primer and probe design

A total of 16 primer-probe sets were used to detect pathogens that certainly or possibly cause BRDC. Each primer-probe set was designed to detect a single target pathogen. As shown in Table 1, seven primer-probe sets were adopted from a previously reported real-time PCR assay [2, 18, 20, 21, 26, 32, 33]. The remaining nine primer-probe sets were newly designed in this study. To design these primers and probes, nucleotide sequences obtained from GenBank of a target pathogen were aligned, and then, primers and probes were selected using PrimerQuest software (Integrated DNA Technologies, Inc., Coralville, IA, U.S.A.). Accession numbers of sequences used for primer-probe design are listed in Supplemental Table 1. The specificities of primers and probes were checked by BLAST search. In addition, as described in a previous report, a primer-probe set for bovine β-actin was as an internal control of RNA/DNA extraction and PCR reaction [31, 32]. TaqMan probes had 6-FAM (6-carboxy-fluorescein) fluorescent reporter molecules at the 5′ ends and TAMRA (6-carboxy-tetra-methyl-rhodamine) quencher molecules at the 3′ ends. TaqMan probes targeting BPIV-3 and *U. diversum* contained 6-FAM at the 5′ ends and MGB (minor groove binder) and NFQ (non fluorescent quencher) at the 3′ ends. Primers and probes were custom-synthesized at Sigma-Aldrich (Sigma Aldrich, St. Louis, MO, U.S.A.) and MGB probes at Applied Biosystems (Applied Biosystems, Foster City, CA, U.S.A.). The information on primers and probes is outlined in Table 1.

DNA and RNA extraction

Nucleic acids were extracted using the following four commercial kits according to the manufacturers' instructions. Virus, bacteria and negative nasal swab sample used for the sensitivity test were centrifuged at 14,000 ×g for 5 min. Viral DNA and RNA were extracted from supernatants, using a High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Mannheim, Germany), with an elution volume of 25 *µl*. Bacterial DNA was extracted from cell pellets, using a QIAamp UCP Pathogen Mini Kit (Qiagen, Hilden, Germany) with a sample volume of 1 m*l* and elution volume of 50 *µl*. In extraction of clinical samples, RNA was extracted using a QIAamp Viral RNA Mini Kit (Qiagen) with a sample volume of 140 *µl* and elution volume of 60 *µl*. DNA was extracted using a QIAamp DNA Mini Kit (Qiagen) with a sample volume of 200 *µl* and elution volume of 200 *µl*. The extracted DNA and RNA were stored at −80°C until it was tested by real-time PCR.

Real-time PCR

A One Step PrimeScript RT-PCR Kit (Perfect Real Time) (TaKaRa Bio, Otsu, Japan) was used to detect viral RNA, and Premix Ex Taq (Perfect Real Time) (TaKaRa Bio) was used to detect viral and bacterial DNA. Real-time PCR assays were conducted in the same reaction conditions, including reaction mix components and thermal cycling, as those used in the Dembo diarrhea-PCR [31]. Fluorescent signal data were analyzed using an automatic quantification algorithm in LightCycler Nano Software 1.1 (Roche Diagnostics GmbH), and the parameters of analysis were as follows: exclude early cycle=7, minimum relative amplifications=0, and minimum amplification quality=5.

Validation of real-time PCR performance using synthesized DNA

To verify the sensitivity, linearity and efficiency of the real-time PCR assay, the limit of detection (LOD), correlation coefficient (R^2) and PCR efficiency (E) were determined from standard curves. Standard curves were obtained, and the LOD, R^2 and E were calculated as described previously [31].

Sensitivity test for nasal swabs

In a previous study, nasal swab samples appeared to contain a PCR inhibitor that yielded false negative results [25]. To evaluate

Table 1. The primer-probe sets used for the detection system

Target pathogen	Target gene		Primer/Probe sequence 5′–3′	Product size (bp)	Reference No.
Bovine viral diarrheal virus	5′UTR	F	GGGNAGTCGTCARTGGTTCG	190	[20]
		R	GTGCCATGTACAGCAGAGWTTTT		
		P	FAM-CCAYGTGGACGAGGGCAYGC-TAMRA		
Bovine coronavirus	Nucleocapsid	F	GGACCCAAGTAGCGATGAG	90	This study
		R	GACCTTCCTGAGCCTTCAATA		
		P	FAM-ATTCCGACTAGGTTTCCGCCTGG-TAMRA		
Bovine parainfluenza virus 3	matrix (M) protein	F	TGTCTTCCACTAGATAGAGGGATAAAATT	90	[18]
		R	GCAATGATAACAATGCCATGGA		
		P	FAM-ACAGCAATTGGATCAATAA-NFQ-MGB		
Bovine respiratory syncytial virus	Nucleocapsid	F	GCAATGCTGCAGGACTAGGTATAAT	124	[2]
		R	ACACTGTAATTGATGACCCCATTCT		
		P	FAM-ACCAAGACTTGTATGATGCTGCCAAAGCA-TAMRA		
Influenza D virus	PB1	F	CAGCTGCGATGTCTGTCATAAG	83	This study
		R	ACAAATTCGCAGGGCCATTA		
		P	FAM-AATGGACTTTCTCCTGGGACTGCT-TAMRA		
Bovine rhinitis A virus	3Dpol	F	CACCTGAACTATGGACTTGG	171	This study
		R	CACGGCCTCAATCATCTG		
		P	FAM-GACGTGGACTGGCACCAGTTTGC-TAMRA		
Bovine rhinitis B virus	3Dpol	F	AACGCGATTGTGTCCTAGGG	112	This study
		R	GCCACTGAGGTTAGCTTCTC		
		P	FAM-CTGTCCTTTGCACGGCGTGG-TAMRA		
Bovine herpesvirus 1	gE	F	CAATAACAGCGTAGACCTGGTC	85	[32]
		R	GCTGTAGTCCCAAGCTTCCAC		
		P	FAM-TGCGGCCTCCGGGCTTTACGTCT-TAMRA		
Bovine adenovirus 3	Hexon	F	ATTACCAGCGTCAACCTCTAC	121	This study
		R	CCGCCGAGAGATAGTCATTAAA		
		P	FAM-TCCACTTTGGAAGCTATGCTCCGC-TAMRA		
Bovine adenovirus 7	Hexon	F	CRAGGGAATAYYTGTCTGAAAATC	87	[33]
		R	AAGGATCTCTAAATTTYTCTCCAAGA		
		P	FAM-TTCATCWCTGCCACWCAAAGCTTTTTT-TAMRA		
Mannheimia haemolytica	sodA	F	ATTAGTGGGTTGTCCTGGTTAG	144	This study
		R	GCGTGATTTCGGTTCAGTTG		
		P	FAM-CTGAACCAACACGAGTAGTCGCTGC-TAMRA		
Pasteurella multocida	kmt-1	F	GGGCTTGTCGGTAGTCTTT	148	This study
		R	CGGCAAATAACAATAAGCTGAGTA		
		P	FAM-CGGCGCAACTGATTGGACGTTATT-TAMRA		
Histophillus somni	16S-rRNA	F	AAGGCCTTCGGGTTGTAAAG	93	This study
		R	CCGGTGCTTCTTCTGTGATTAT		
		P	FAM-CGGTGATGAGGAAGGCGATTAG-TAMRA		
Trueperella pyogenes	plo-Pyolysin	F	ATCAACAATCCCACGAAGAG	99	This study
		R	TTGCAGCATGGTCAGGATAC		
		P	FAM-TCGACGGTTGGATTCAGCGCAATA-TAMRA		
Mycoplasma bovis	oppD	F	TCAAGGAACCCCACCAGAT	71	[26]
		R	AGGCAAAGTCATTTCTAGGTGCAA		
		P	FAM-TGGCAAACTTACCTATCGGTGACCCT-TAMRA		
Ureaplasma diversum	16S-rRNA	F	CATTAAATGATGTGCCTGGGTAGTAC	61	[21]
		R	CCCCGTCAATTCCGTTTG		
		P	FAM-TTCGCAAGAATGAAAC-NFQ-MGB		
β-ACTIN	Actin	F	AGCGCAAGTACTCCGTGTG	96	[32]
		R	CGGACTCATCGTACTCCTGCTT		
		P	FAM-TCGCTGTCCACCTTCCAGCAGATGT-TAMRA		

F: Forward primer; R: Reverse primer; P: Probe.

PCR inhibition by nasal swab components, a positive control nucleic acid was added to negative samples after nucleic acids extraction as previously described [3, 24]. Nasal swabs collected from clinically healthy cattle that were confirmed to be negative by Dembo respiratory-PCR for all target pathogens were tested. These swabs were suspended in phosphate-buffered saline (−) to make 10% emulsion. Isolated strains of BPIV-3 strain BN-1 (AB770484), BRSV strain NMK-7, BRAV strain H-1 (JN936206), *M. haemolytica* strain N791 (bovine/Japan/1979), *P. multocida* strain TS-8, *H. somni* strain 23N2359, *T. pyogenes* strain 42 and

Table 2. Performance of sensitivity tests based on nasal swab samples

Type of spiked nucleic acid	Pathogens	LOD (/reaction)	Reproducibility CV (%)
Viral DNA or RNA ($TCID_{50}$)	Bovine viral diarrheal virus	1	0.67
	Bovine parainfluenza virus 3	100	2.25
	Bovine respiratory syncytial virus	0.1	0.30
	Bovine rhinitis A virus	0.1	1.30
	Bovine herpesvirus 1	10	0.09
	Bovine adenovirus 7	1	0.66
Bacterial DNA (CFU)	*Mannheimia haemolytica*	1	0.41
	Pasteurella multocida	1	0.06
	Histophillus somni	0.1	0.26
	Trueperella pyogenes	100	2.06
	Mycoplasma bovis	1	1.42
Synthesized DNA (copies)	Bovine coronavirus	10	1.34
	Influenza D virus	10	0.25
	Bovine rhinitis B virus	100	0.89
	Bovine adenovirus 3	10	0.99
	Ureaplasma diversum	10	1.62

LOD: Limit of detection; CV: Coefficient of variation.

M. bovis strain Donetta were obtained from the National Veterinary Assay Laboratory in Japan. The BVDV strain Nose (bovine / Japan/1974) and BHV-1 strain Ishikawa (bovine /Japan/1988) used in this study were isolated from field samples. To evaluate the primer-probe sets targeting BCoV, IDV, BRBV, BAdV 3 and *U. diversum*, synthesized DNA (Integrated DNA Technologies, Inc.) was used instead of nucleic acids extracted from infectious pathogens, because these pathogens were unavailable. Nucleic acids from the 10% swab emulsion and pathogens (1.0×10^5 $TCID_{50}$/m*l* or CFU/m*l*) were extracted as described above. Then, 10-fold serial dilutions of the extracted nucleic acids or synthesized DNA were spiked into the eluates of negative nasal swabs. The final concentrations were adjusted to the following: pathogen DNA or RNA, 1.0×10^{-1} to 1.0×10^2 $TCID_{50}$/reaction or CFU/reaction; and synthesized DNA, $1.0 \times 10^{\circ}$ to 10^3 copies/reaction. The tests were performed in duplicate using separate serial dilutions. The LOD was determined, and reproducibility was evaluated using the coefficient of variation (CV) calculated from the quantification cycle (Cq).

Dembo respiratory-PCR assay of clinical samples

The assay was applied to test clinical samples; we also evaluated the diagnostic performance of the assay. A total of 40 samples of bovine nasal swabs were collected in 2015 from six farms (farms A–F) in which BRDC pathogens presences were identified in Gifu Central Livestock Hygiene Service Center. Information of individual cattle were indicated in Supplemental Table 2. Nucleic acids extracted from samples were tested in triplicated with Dembo-PCR [31]. Samples were determined positive, if the Cq values were calculated by the algorithm described above in more than two of three run.

RESULTS

Sensitivity, linearity and efficiency evaluated with standard curves from synthesized DNA

To evaluate the sensitivity, linearity and efficiency of the PCR, 10-fold serial dilutions of synthesized DNA were tested by real-time PCR. Standard curves were constructed from Cq values, and then, the LOD, R^2 and E were evaluated (Fig. S1). For BVDV, BHV-1 and BAdV 7, standard curves have already been evaluated under the same conditions in a previous study [31]. The LOD, based on DNA copy number, was ≤100 copies/reaction for all primer-probe sets. In addition, the calibration curves of all assays covered a linear dynamic range of more than five orders of magnitude and showed R^2 values of at least 0.9818. PCR efficiencies were in the range of 84.2–101.8%.

Sensitivity and reproducibility tests using nasal swab samples

The LOD, taking into account possible PCR inhibition by components of nasal swab samples, and CV values, used to evaluate inter-assay error, are described in Table 2. Except for that of BPIV-3, all LODs were ≤10 $TCID_{50}$/reaction for extracted nasal swabs spiked with viral nucleic acids. For bacterial nucleic acids, the LOD of the assay for all bacteria other than *T. pyogenes* was ≤1 CFU/reaction. The LOD of *T. pyogenes* was higher than those for other bacteria. For the synthesized DNA, because the pathogens were not available, an LOD of ≤100 copies/reaction was also obtained in each assay. CV values were at most 2.25%; this reproducibility was observed with BPIV-3 testing.

Table 3. Result of Dembo respiratory-PCR using clinical samples

Pathogens	Positive samples in Dembo respiratory-PCR						
	Farm A N=8 n (%)	Farm B N=6 n (%)	Farm C N=4 n (%)	Farm D N=5 n (%)	Farm E N=7 n (%)	Farm F N=10 n (%)	Total N=40 n (%)
Bovine viral diarrheal virus	-	1 (16.7)	-	-	-	-	1 (2.5)
Bovine coronavirus	-	5 (83.3)	-	5 (100)	-	-	10 (25.0)
Bovine parainfluenza virus 3	-	-	-	-	-	-	-
Bovine respiratory syncytial virus	7 (85.7)	1 (16.7)	-	-	5 (71.4)	10 (100)	23 (56.4)
Influenza D virus	-	-	-	-	-	-	-
Bovine rhinitis A virus	-	-	-	-	-	-	-
Bovine rhinitis B virus	-	4 (66.7)	-	1 (20.0)	6 (85.7)	-	11 (27.5)
Bovine herpesvirus 1	-	-	-	-	-	-	-
Bovine adenovirus 3	-	-	-	-	-	-	-
Bovine adenovirus 7	-	-	-	-	-	-	-
Mannheimia haemolytica	2 (25.0)	4 (66.7)	2 (50.0)	3 (60.0)	-	1 (10.0)	12 (30.0)
Pasteurella multocida	8 (100)	6 (100)	4 (100)	5 (100)	-	-	23 (57.5)
Histophillus somni	4 (50.0)	5 (83.3)	4 (100)	4 (80.0)	-	1 (10.0)	18 (45.0)
Trueperella pyogenes	-	-	3 (75.0)	2 (40.0)	1 (14.3)	1 (10.0)	7 (17.5)
Mycoplasma bovis	6 (75.0)	5 (83.3)	4 (100)	5 (100)	-	-	20 (50.0)
Ureaplasma diversum	6 (75.0)	5 (83.3)	4 (100)	4 (80.0)	2 (28.6)	2 (20.0)	24 (60.0)

Dembo respiratory-PCR performance in clinical sample testing

A total of 40 nasal swabs from different affected animals on six farms with BRDC outbreaks were applied to Dembo respiratory-PCR. The results are presented as the number and percentage of positive samples from each farm (Table 3). In samples from farm A, both viral and bacterial pathogens, including BRSV (85.7%), *P. multocida* (100%), *M. bovis* (75%) and *U. diversum* (75%), were detected at high frequency, whereas mainly viral pathogens, including BRSV (71.4%) and BRBV (85.7%) in farm E and BRSV (100%) in farm F, were prevailed. In samples from farms B and D, mixed infections of BCoV (83.3% and 100%) and bacterial pathogens were detected. The results of tests of individual cattle are shown in Supplemental Table 2.

DISCUSSION

In this study, Dembo respiratory-PCR was developed, following the methods applied for Dembo diarrhea-PCR described in a previous report [31]. Since all primer-probe sets were optimized in the same temperature conditions, Dembo respiratory-PCR can detect a total of 16 pathogens, including 10 viruses and six bacteria, in a single run of TaqMan real-time PCR. Notably, we designed primers for IDV, BRAV and BRBV, which are not traditionally tested in Japan as etiologic agents of BRDC. IDV was first found in pigs and then from cattle, showing a significant association with BRDC [14, 23]. An assay to detect IDV by TaqMan real-time PCR has already been reported [15]. However, new IDV strains were recently identified [4–6, 19], and the nucleic acid sequences of some strains have mismatches to the reported primer-probe set. To address this issue, a new primer-probe set was designed in this study. Nevertheless, forward primer has a few mismatches to the sequence of D/bovine/Ibaraki/7768/2016, which was identified very recently, and the primer is needed for further improvement. BRAV and BRBV have been commonly found in cattle with BRDC in the United States [13]. BRBV was detected from BRDC cattle also in this study, but a previous study suggested that BRBV was not significantly associated with BRDC [23]. The importance of BRBV in BRDC remains uncertain. IDV and BRAV were undetected in the clinical samples in this study. In sensitivity test, all assays showed low LOD (≤100 CFU, $TCID_{50}$ or copies/reaction). The LOD of BPIV-3 was higher than other virus (100 $TCID_{50}$/reaction), but a previous study showed a similar level of sensitivity [18]. Dembo respiratory-PCR can be performed to detect pathogens that are currently considered to be associated with BRDC.

By Dembo respiratory-PCR, multiple BRDC pathogens that infect cattle can be detected comprehensively and simultaneously. The Dembo respiratory-PCR can quickly elucidate combinations of pathogen in a sample. Notably, in one BVDV-positive sample, BCoV, BRSV and bacterial pathogens were also detected. A previous study indicated that BRSV infection diminished the host's clearance ability in the upper respiratory tract or altered the immune response [9, 10], possibly facilitating bacterial opportunistic infections, whereas an experimental study showed that infection with BRSV infection alone resulted in clinical signs and lesions in lung tissue [10]. As well as BRSV, BCoV generally impairs mucosal immunity in the upper respiratory tract [27]. In this way, the Dembo respiratory-PCR helps revealing the infection of multiple pathogens at once. For the same purpose, multiplex PCRs that can detect utmost three pathogens were developed in the previous studies [18, 30]. However, multiplex PCR has the restriction on target number and the risk that primer-probe sets interfere in each other.

Compared with one assay-one pathogen test, Dembo respiratory-PCR can identified a wide range of existing pathogens quickly

and easily. Considering multiple etiology of BRDC, screening by Dembo respiratory-PCR would help determining treatment and prevention measures. This detection system may provide an alternative testing method that is simpler, earlier and more comprehensive than existing assays.

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