Research Article

Development of a Taqman Real-Time PCR Assay for Detection of *Bordetella bronchiseptica*

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Abstract | Infection with *Bordetella bronchiseptica* is the most common bacterial cause of kennel cough or canine infectious respiratory disease (CIRD) in dogs. Other disease agents include various respiratory viruses and co-infection can exacerbate the clinical signs. CIRD is highly contagious and can spread rapidly, especially among dogs in close quarters. Fast and accurate diagnostics enables the selection of proper management. Our objective was to develop a sensitive and specific real-time PCR for detection of *B. bronchiseptica* in samples from dogs with respiratory signs. A genome comparison program was used to select a suitable target DNA sequence. A PCR targeting the *bfrZ* gene was developed which showed no cross-reactivity *in silico* or when tested with a panel of bacterial isolates. The limit of detection was determined to 4×10^3 bacteria per mL of nasal swab sample and less than 10 copies of target DNA per PCR reaction. Out of 23 isolates of *B. bronchiseptica* tested, one isolate from a hedgehog was not amplified. Sequencing of the 16S rDNA showed that the isolate was similar to strains of both *B. bronchiseptica* and *B. parapertussis* but was considered to be of minor importance for the diagnostics of dogs. In a panel of culture-negative nasopharyngeal swabs from dogs with (n=57) or without (n=17) clinical signs of CIRD, three resulted PCR-positive for *B. bronchiseptica*. Altogether, the novel assay is highly specific and sensitive for detection of *B. bronchiseptica* in clinical samples.

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Introduction

Bordetellae are gram-negative ß-proteobacteria that colonize the respiratory tract of mammals. *B. bronchiseptica* is the most common bacteriological cause of kennel cough or canine infectious respiratory disease (CIRD), a highly contagious and multifactorial upper respiratory syndrome of dogs. Other primary causes of CIRD include canine parainfluenzavirus, canine respiratory coronavirus (Mitchell et al., 2013), as well as other viruses (Buonavoglia and Martella, 2007; Mitchell et al., 2013, Schulz et al., 2014). Healthy dogs can harbor *B. bronchiseptica*, and following clinical signs of CIRD caused by *B. bronchiseptica*, dogs may shed the bacteria for up to three months, which makes it difficult to eradicate (Bemis et al., 1977). Clinical signs include dry cough, tracheal mucus accumulation, vomiting and pneumonia, and usually last for 1-3 weeks after which a temporary immunity develops (Goodnow, 1980; Greene, 2006). The traditional diagnostic method consists of culturing nasopharyngeal swabs on non-selective and differentiating growth media, which may take several days and depends on large amounts of bacteria for

successful isolation, followed by biochemical testing. Real-time PCR is a fast and sensitive method suitable for detection of specific pathogens. Previous work by others have aimed at developing methods that could distinguish several different *Bordetella*-species, targeting multi-copy insertion sequences present in more than one species (Roorda et al., 2011; Tatti et al., 2011). These methods demand running several assays per sample (Tizolova et al., 2014) and the use of algorithms to deduce proper results. Others have developed similar assays based on SYBR Green detection and melt curve analysis and thorough evaluation of any assay has only been done using human clinical isolates (Koidl et al., 2007; Roorda et al., 2011; Tatti et al., 2011; Tizolova et al., 2014). A SYBR Green real-time PCR was developed recently for detection of *B. bronchiseptica* in dogs (Schulz et al., 2014), but this assay was deemed unspecific as it cross-reacts with sequences of *B. parapertussis* when tested *in silico*. Depending on the purpose of diagnostics this might not be a problem. However, the objective of the present study was to develop a sensitive and specific Taqman based real-time PCR for direct detection of *B. bronchiseptica* in canine samples, suitable for combination with real-time RT-PCR for other canine respiratory pathogens.

Material and Methods

DNA extraction

DNA was extracted from bacterial cultures by suspension of roughly 1 µL colony material in 500 µL nuclease free water followed by boiling at 98-100°C for ten minutes. The lysates were rapidly chilled in ice and then centrifuged at 13.000 × *g* for five minutes. The supernatant was used as PCR template. ESwabs with Amie's medium (Copan diagnostics), used to collect clinical specimens, were shaken thoroughly and 90 µL of the liquid content was mixed with 10 µL proteinase K, 20 mg/mL (Sigma-Aldrich) and incubated at room temperature for 5 minutes prior to DNA extraction using Magnatrix 8000+ automatic platform and Vet Viral NA kit (NorDiag AB) according to the manufacturer's instructions.

Real-time PCR assay design

To find potential PCR targets within the *B. bronchiseptica* genome that would amplify *B. bronchiseptica,* but not other *Bordetellae,* Gegenees software V2.2.1 for comparative genome analysis was used (available from http://www.gegenees.org/). The following whole genome sequences were used for assay design: *B. bronchiseptica* 253 (NC_019382.1), *B. bronchiseptica* MO149 (NC_018829.1), *B. bronchiseptica* RB50 (NC_002927.3), *B. bronchiseptica* KM22 (JMHR01000000), *B. bronchiseptica* 1289 (NZ_CAKS00000000.1), *B. parapertussis* Bpp5 (NC_018828.1), *B. parapertussis* 12822 (NC_002928.3), *B. parapertussis* 18323 (NC_018518.1), *B. pertussis* Tohama I (NC_002929.2), *B. pertussis* CS (NC_017223.1), *B. avium* 197N (NC_010645.1) and *B. petrii* ASM6720v1 (NC_010170.1). A real-time PCR assay was subsequently designed targeting the *bfrZ* gene, present in one copy in the *B. bronchiseptica* genome, using a primer set suggested by the software and a DNA probe that hybridizes to the PCR product which was labeled with carboxyfluorescein (FAM) at the 5'-end and a non-fluorescent quencher (BHQ1) at the 3'-end (Table 1). Sequence specificity and sensitivity of the assay were evaluated *in silico* by basic local alignment search tool, Primer-BLAST (http://www.ncbi.nlm.nih.gov/). The PCR assay was tested and evaluated in 15 µL PCR reactions containing nuclease free water, PerfeCTa qPCR Toughmix with low ROX reference dye (Quanta Biosciences), 400 nM of each primer, 133 nM FAM-labeled probe and 2 µL template DNA. A 96-well format thermocycler,

Table 1: *Oligonucleotides used for real-time PCR and sequencing in the present study.*

Oligonucleotide	Sequence (5'-3')	Size (bp)	Tm (C°)	Amplicon size (bp)
Real-time PCR				
bfrZ-Qf	CGGAGTGAGATCGTGCATCA	20	59	182
bfrZ-Qr	CCACCAAACGCAATGACCTG	20	59	
bfrZ-Qp	FAM-TCGGGAAGGTGCAGCATGTCCTGGAAA-TA-BHQ1	29	68	
Sequencing				
fD1/2	AGAGTTTGATCMTGGCTCAG	20	56	1494
rP1	ACGGTTACCTTGTTACGACTT	21	56	

Table 2: *Evaluation of the bfrZ PCR assay on clinical isolates and reference strains of Bordetella. Also shown are the Maldi-TOF results, appearance on Smith-Baskerville agar and oxidase reaction with tetramethyl-p-phenylenediamine (TMPD).*

	ID	Source / type of material		Real-time PCR *bfrZ*	Maldi-TOF	SB-agar	Oxidase
Reference strains	*B. bronchiseptica*	CCUG 219		+	ND	ND	ND
	B. pertussis	ATCC 9797		-	ND	ND	ND
	B. parapertussis	ATCC 15237		-	ND	ND	ND
Clinical isolates	07-BKT014491	-	-	+	*B. bronchiseptica*	blue	+
	07-BKT009522	cat	nasal swab	+	*B. bronchiseptica*	blue	+
	08-BKT049540	horse	air sacs	+	*B. bronchiseptica*	blue	+
	09-VIR118062	dog	nasal swab	+	*B. bronchiseptica*	blue	+
	10-PAT006082	swine	lung	+	*B. bronchiseptica*	blue	+
	10-BKT054754	rabbit	throat swab	+	*B. bronchiseptica*	blue	+
	10-BKT043563	swine	lung QA	+	*B. bronchiseptica*	blue	+
	10-PAT001274	rabbit	lung	+	*B. bronchiseptica*	blue	+
	10-BKT011120	dog	throat swab	+	*B. bronchiseptica*	blue	+
	12-BKT000746	swine	nasal swab QA	+	*B. bronchiseptica*	blue	+
	13-BKT085771	swine	nasal swab QA	+	*B. bronchiseptica*	blue	+
	13-VLT003007	hedgehog	lung	-	*B. bronchiseptica*	blue	+
	13-BKT055188	dog	tracheal aspirate	+	*B. bronchiseptica*	blue	+
	13-BKT054629	brown rat	nasal swab	+	*B. bronchiseptica*	blue	+
	13-BKT001249	dog	nasal swab	+	*B. bronchiseptica*	blue	+
	14-BKT016044	dog	nasal swab	+	*B. bronchiseptica*	blue	+
	14-BKT080448	rabbit	nasal swab	+	*B. bronchiseptica*	blue	+
	15-BKT008354	swine	QA	+	*B. bronchiseptica*	blue	+
	15-VIR120163	horse	lung wash	+	*B. bronchiseptica*	blue	+
	15-BKT070337	horse	nasal wash	+	*B. bronchiseptica*	blue	+
	15-BKT072397	cat	nasal swab	+	*B. bronchiseptica*	blue	+
	15-BKT074975	cat	nasal swab	+	*B. bronchiseptica*	blue	+
	15-BKT075176	dog	nasal swab	+	*B. bronchiseptica*	blue	+

ND=*not done;* **QA**=*isolate from quality assurance testing*

ABI 7500 Fast (Applied Biosystems), was used for amplification and detection of the target sequence. Cycling conditions were as follows: 45°C for 10 minutes, 95°C for 10 minutes, 48 cycles of 95°C for 15 seconds and 60°C for 45 seconds during which fluorescence intensity was measured. Ramp rate was set to 1.6 degrees per second.

Assay validation

A total of 23 clinical isolates and one reference strain of *B. bronchiseptica* were used in the present evaluation (Table 2). Clinical strains had previously been isolated from various animal species during routine diagnostic work at the National Veterinary Institute, Sweden. All clinical isolates were typed as *B. bronchiseptica* by Maldi-TOF (Bruker Daltonik GmbH, Germany) according to the manufacturer's instructions (Table 2). Amplification efficiency and limit of detection were determined by six replicates of ten-fold and two-fold serial dilutions of *B. bronchiseptica* strain CCUG 219 DNA. DNA concentration was measured by Qubit fluorometer using the QuantIT HS kit (Invitrogen) and number of genome copies was calculated as described previously (Mendoza-Cano and Sánchez-Paz 2013), assuming a genome size of 5.26 Mb. Limit of detection in clinical samples was tested by adding reference strain *B. bronchiseptica* CCUG 219 in serial dilutions to ESwabs (COPAN Diagnostics) collected from the nasopharynx of healthy dogs. Swabs were then processed as described above. Viable count was determined by plating dilution series of *B. bronchiseptica* on blood agar plates incubated at 37°C for 48 h, whereupon colonies were counted. Plates containing between 5-250 colonies were included in final

Table 3: *Exclusion panel for the testing of specificity. All isolates tested negative with the bfrZ real-time PCR*

Bacillus cereus CCUG 7414	Haemophilus influenzae ATCC 49247	Proteus mirabilis_SLV-374
Bacillus cereus_B. cereus	Haemophilus somnus CCUG 28029	Pseudomonas aeruginosa CCUG 17619
Bacillus subtilis ATCC 6633	Klebsiella oxytoca CCUG 15717	Pseudomonas aeruginosa_SLV-395
Bacteroides fragilis ATCC 25285	Klebsiella pneumonaie_SLV-186	Pseudomonas aeruginosa_SLV-453
Campylobacter coli_SLV-271	Klebsiella pneumoniae CCUG 225	Staphylococcus aureus CCUG 4151
Campylobacter jejuni_SLV-542	Listeria ivanovii_SLV-348	Staphylococcus aureus_SLV-438
*E. coli*_B266	Listeria monocytogenes CCUG 15527	Staphylococcus intermedius CCUG 49053
*E. coli*_L278	Listeria monocytogones_SLV-513	Staphylococcus xylosus_SLV-283
*E. coli*_S262	Mycoplasma hemofelis BKT75878/14	Streptococcus agalactiae CCUG 39325
*E. coli*_U226	Mycoplasma hyopmneumoniae MS53	Streptococcus dysgalactiae CCUG 27436
*E. coli*_XL-1 blue	Mycoplasma hyorhinis Henri 1/5	Streptococcus equi CCUG 27367
*Enterobacter cloceae*_SLV-011	Mycoplasma pulmonis M672/82	Streptococcus pyogenes CCUG 12701
*Enterococcus duran*_SLV-078	Pasteurella multocida CCUG 229	Streptococcus zooepidemicus CCUG 23256
Enterococcus fecalis ATCC 29212	Pasteurella pneumotropica CCUG 12398	Taylorella asinigenitalis BKT20490/11
Escherichia coli ATCC 35218	Proteus mirabilis CCUG 26767	Taylorella equigenitalis CCUG 16464

Table 4: *Similarity between the 16S rDNA sequence of isolate 13-VLT003007 and strains of B. bronchiseptica and related species based on BLAST search against the Greengenes and GenBank databases*

Identity (%)	Strain name	Origin	Match length (bp)	Accession nr.
100.00	*Bordetella bronchiseptica* RB50	rabbit	1392	BX640442.1
100.00	*Bordetella bronchiseptica* 253	dog	1392	HE965806.1
100.00	*Bordetella bronchiseptica* S798	swine	1392	AP014582.1
100.00	*Bordetella bronchiseptica* MO149	human	1392	HE965807.1
100.00	*Bordetella bronchiseptica* NBRC 13691	dog	1392	NR_113628.1
100.00	*Bordetella bronchiseptica* Bb1	rabbit	1365	EU643519.1
100.00	*Bordetella parapertussis* 12822	human	1392	BX640434.1
100.00	*Bordetella parapertussis* B24	human	1392	AF366577.1
100.00	*Bordetella bronchiseptica* S-1 pig	swine	1392	X57026.1
99.93	*Bordetella bronchiseptica* ATCC 4617	-	1392	AJ278452.1
99.86	*Bordetella bronchiseptica* Eq 128	horse	1392	KT368942.1
99.93	*Bordetella parapertussis* DSM 4922	-	1392	AJ278450.1
99.86	*Bordetella parapertussis* B1232	sheep	1388	AF366578.1
99.85	*Bordetella bronchiseptica* Bb3	rabbit	1366	EU643521.1
99.78	*Bordetella pertussis* B3621	human	1392	CP011401.1
99.50	*Bordetella parapertussis* 522; ATCC 15311	human	1392	NR_025950.1
99.35	*Bordetella bronchiseptica* S1	-	1392	E06073.1
99.35	*Bordetella bronchiseptica* Dog 71; ATCC 19395	dog	1392	NR_025949.1

calculations of CFU (colony forming unit) per mL of sample. To test assay specificity a broad-range exclusion panel of 45 bacterial isolates (Table 3), plus reference strains of *B. pertussis* and *B. parapertussis* (Table 2), were analyzed. These bacteria were chosen as they either represent the normal flora of canine upper respiratory tract (Bailie et al., 1978; Elliott et al., 2005) or are genetically closely related to *B. bronchiseptica*. To evaluate the PCR assay in clinical samples from dogs, 74 nasopharyngeal swabs collected from 57 dogs with clinical signs of CIRD and 17 dogs without signs of CIRD were used. All samples had been culture negative for *B. bronchiseptica* in routine diagnostic investigations.

16S rDNA sequencing

Amplification of 16S rDNA was done as described by Weisburg et al. (1991), with slight modifications; PCR reaction contained 2 µL template DNA, Sso-Fast™ EvaGreen® Supermix with Low ROX (Bio-

Rad) and 250 nM of each primer (Table 1) with following cycling conditions: 95˚C for 3 minutes followed by 35 cycles of 95˚C for 30 seconds, 42˚C for 30 seconds and 72˚C for 4 minutes, followed by 72˚C for 20 minutes. PCR products were verified by melt curve analysis: 95˚C for 15 seconds, 60˚C for 60 seconds, temperature increase from 60˚C to 95˚C by a 1% ramp rate, 95˚C for 15 seconds and finally 60˚C for 15 seconds. PCR product purification was performed by incubating 10 µL PCR product with 0.05 µL ExoI and 0.5 µL FastAP (Thermo Scientific) for 15 minutes at 37ºC followed by inactivation for 15 minutes at 85ºC. A mixture of 5 µL purified PCR product and 50 pmol of either forward or reverse primer was sent for Sanger sequencing (Macrogen, The Netherlands). Sequences were assembled using CLC Main Workbench V6.7 and compared to the NCBI 16S rDNA sequence database (nucleotide BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results and Discussion

Assay design

For initial design of a real-time PCR assay, whole genome sequences of five *B. bronchiseptica* strains were used. These strains represented all four clusters of *flaA* types (1a, 1b, 2 and 3) described by Khayer *et al.*, (Khayer et al., 2014) and have been isolated from different host species (dog, human, rabbit, pig and monkey). Typing of the *flaA* gene, which encodes a subunit of the flagellar filaments, has been used to detect genetic diversity in a number of bacterial species, for instance *Campylobacter spp., Legionella pneumophila* and *Bordetella spp.* (Fitch et al., 2005; Edwards et al., 2008; Khayer et al., 2014). The aim was to find a potential PCR target region that was sufficiently conserved even among a diverse set of isolates within the species. Related *Bordetella* species were used to exclude unspecific regions. The specificity and sensitivity of the potential target region was then verified *in silico* by Primer-BLAST before continuing on with laboratory testing.

Validation

For validation of the *bfrZ* real-time PCR we tested detection limit, amplification efficiency, intermediate precision, sensitivity and specificity. Limit of detection was determined by PCR of serial dilutions of purified DNA of *Bordetella bronchiseptica* CCUG 219 and lowest amount detected in six replicates was 8 copies/reaction. After plotting concentrations against C_T-values, PCR amplification efficiency was calculated to 95% over 7 log (Figure 1).

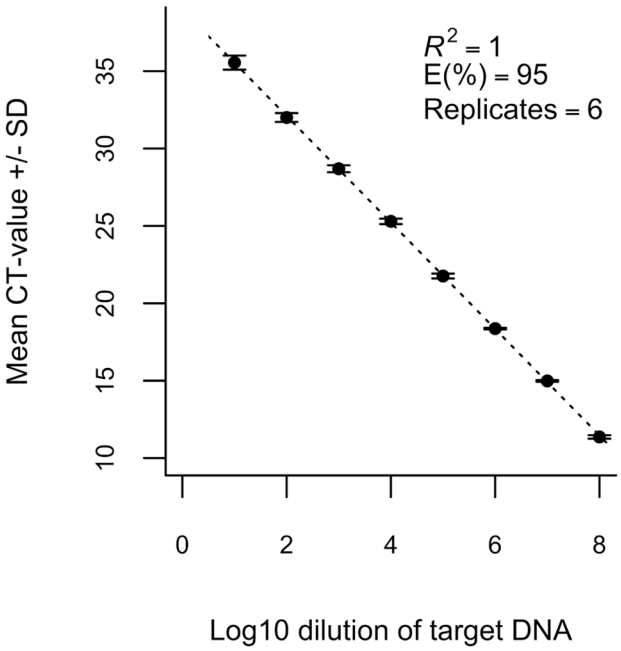

Figure 1: *Amplification efficiency of the bfrZ real-time PCR*

A standard curve was generated by 10-fold serial dilutions of B. bronchiseptica CCUG 219 DNA. PCR in six replicates revealed a good linear relationship (E=95%) and a wide dynamic range.

Intermediate precision was determined by analyzing diluted DNA of CCUG 219 in three replicates in five different PCR runs at different days and coefficient of variance was calculated to 1.6%. In absence of culture positive clinical specimens, diagnostic sensitivity could not be properly evaluated; however, limit of detection in clinical samples was determined by a spike-in experiment. Lowest amount of bacteria detected in clinical samples spiked with *Bordetella bronchiseptica* CCUG 219 in three consecutive experiments was 4×10^3 CFU/mL of sample on average, which corresponds to a theoretical value of less than 10 CFU/PCR reaction, assuming a 100% DNA yield during sample preparation and extraction. Inclusion and exclusion was evaluated by a set of bacterial isolates as well as *in silico*. None of the bacterial isolates in the exclusion panel tested positive, and all but one isolate of *B. bronchiseptica* tested positive by the newly developed PCR assay, which resulted in a sensitivity (inclusion) of 96% and a specificity (exclusion) of 100%. The temperature profile included a reverse transcription step at 45˚C to enable simultaneous amplification and detection of CIRD associated RNA viruses. However, the assay was also tested without this step, with shorter denaturation and elongation times and ramp

rates as fast as 3.5 degrees per second, with similar amplification efficiency and detection limit (data not shown). Next, the assay was evaluated using clinical samples collected from dogs with (n=57) and without CIRD (n=17). Two dogs with signs of CIRD and one dog with no signs of CIRD were found positive for *B. bronchiseptica*. The PCR-positive healthy control dog had been vaccinated with an intra-nasal live attenuated *B. bronchiseptica* vaccine (Nobivac KC vet., Intervet, Sweden) nine days prior to sampling and most likely the PCR assay detected the vaccine strain. It has been shown that the live attenuated vaccine strain of *B. bronchiseptica* is shed during the first month after vaccination (Iemura et al., 2009).

16S rDNA sequencing

All but one isolate were amplified by the *bfrZ* assay (Table 2). Isolate 13-VLT003007, which derived from a hedgehog, was identified as *B. bronchiseptica* by Maldi-TOF but could not be detected with the *bfrZ* PCR and was therefore subjected to 16S rDNA sequencing in order to make a taxonomic analysis. BLAST analysis against 16S sequences at GenBank and Greengenes (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi) showed that 13-VLT003007 was identical to previously sequenced strains of both *B. bronchiseptica* and *B. parapertussis* but also showed differences to several strains of *B. bronchiseptica* (Table 4). This indicates that 13-VLT003007 is closely related to both species but the 16S rDNA sequence probably does not offer sufficient resolution to determine exact taxonomic relationship of such closely related species as *B. bronchiseptica* and *B. parapertussis*.

Conclusions

Here we describe development and validation of a probe-based real-time PCR assay to detect the *bfrZ* gene of *Bordetella bronchiseptica* for diagnosis of respiratory infections in dogs. The assay was evaluated with nasopharyngeal swabs from dogs with and without clinical signs of CIRD. All swab samples had been tested negative for *B. bronchiseptica* with current standard culture methods; however, by the newly developed real-time PCR assay, three of the samples showed positive reaction for *B. bronchiseptica*. Specificity of the assay was evaluated both *in silico* and with an exclusion panel of bacterial isolates and no false positives were detected. There was one false negative among the *B. bronchiseptica* isolates but it was considered to be of minor importance for diagnostics of dogs. In conclusion, the presented real-time PCR assay for *B. bronchiseptica* provides a sensitive and specific detection method, suitable for combination with viral molecular diagnostic assays for determining the etiological cause of CIRD in clinical samples.

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Author's Contributions

TJ was responsible for assay design, experimental design, collection and analysis of data and drafting. KM was involved in data generation and collection. EE was involved in providing clinical specimens and strains and was revising the intellectual content of the manuscript. JJW was involved in providing clinical specimens, revising intellectual content and gave the final approval of the manuscript.

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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