Misidentification of *Bordetella bronchiseptica* as *Bordetella pertussis* using a newly described real-time PCR targeting the pertactin gene

Karen B. Register and Tracy L. Nicholson

Respiratory Diseases of Livestock Research Unit, USDA/Agricultural Research Service/National Animal Disease Center, Ames, IA 50010, USA

Correspondence

Karen B. Register

kregiste@nadc.ars.usda.gov

Received 13 July 2007

Accepted 18 August 2007

Recently, a real-time PCR (RT-PCR) assay based on sequence from the gene for pertactin was proposed for identification of *Bordetella pertussis*. Here, it is reported that the *B. pertussis* pertactin gene sequence for the region that encompasses the RT-PCR probe and primers is nearly identical to that of many *Bordetella bronchiseptica* strains of human and avian origin. Additionally, it is demonstrated that such strains are erroneously identified as *B. pertussis* using the RT-PCR assay. These data suggest that the use of the assay without confirmatory testing may result in erroneous identification of a significant proportion of human isolates of *B. bronchiseptica* as *B. pertussis*.

INTRODUCTION

Bordetella pertussis is the aetiologic agent of whooping cough, an acute respiratory disease that occurs exclusively in humans. A rapid, sensitive and specific method for the identification of *B. pertussis* is crucial for the effective management of outbreaks and to monitor prevalence. The repetitive element IS481 is frequently targeted for rapid detection of *B. pertussis* by PCR (Riffelmann *et al.*, 2005). While assays based on this approach are highly sensitive, the presence of IS481 in other species of *Bordetella* compromises their specificity (Diavatopoulos *et al.*, 2005; Register & Sanden, 2006; Riffelmann *et al.*, 2005; van der Zee *et al.*, 1997). Recently, a novel real-time PCR (RT-PCR) assay that targets the gene for pertactin, a *Bordetella* adhesin and virulence factor, was proposed as an alternative (Vincart *et al.*, 2007). Although the authors concluded that the assay alleviates problems related to cross-reactivity with *Bordetella* species other than *pertussis*, only single isolates of other species appear to have been evaluated. The goals of the present study were to determine the sequence of the relevant region of the pertactin gene from a large number of *Bordetella bronchiseptica* strains, including several of human origin, for comparison with the sequence of *B. pertussis*, and to evaluate the potential for false-positive results using the pertactin RT-PCR.

Abbreviation: RT-PCR, real-time PCR.

The GenBank/EMBL/DDBJ accession numbers for the *Bordetella* sequences determined in this paper are EU031919–EU032001.

A table showing *B. bronchiseptica* isolates used for sequence comparisons is available as supplementary data with the online version of this paper.

METHODS

Bacterial isolates. This study includes data derived from 88 isolates of *B. bronchiseptica*, obtained from at least 11 different host species. Initial identification of isolates was based on colony morphology and standard biochemical testing, and was confirmed by subsequent ribotype analysis (Register *et al.*, 1997). Information related to individual isolates is available in Supplementary Table S1. *B. pertussis* Tohama I was used as a positive control in the pertactin RT-PCR. All isolates were grown at 37 °C for 36 h (*B. bronchiseptica*) or 72 h (*B. pertussis*) on Bordet–Gengou agar supplemented with 10% sterile, defibrinated sheep's blood.

PCR and sequence analysis. Chromosomal DNA was purified with a commercially available kit (Gentra Systems) and quantified with PicoGreen (Molecular Probes). The Primer Design module of Vector NTI Advance (Invitrogen) was used to select PCR primers for amplification of the pertactin gene region of interest (forward, 5'-GATGATGCGTCGCTGTAACA-3'; reverse, 5'-AGGGTGCCGATATGCAAGCC-3'). PCR was carried out using 100 ng purified DNA and the reaction components and cycling conditions described previously (Register, 2004). Five microlitres of each reaction was analysed by agarose gel electrophoresis in 3:1 NuSieve (Cambrex BioScience) containing 0.5 µg ethidium bromide ml⁻¹. Amplicons were purified using spin columns (Qiagen), and were sequenced directly by fluorescence-based cycle sequencing with AmpliTaq and BigDye Terminators on an ABI 377 sequencer, at the National Animal Disease Center Genomics Unit. Sequences were analysed using Vector NTI Suite software.

RT-PCR. RT-PCR was carried out using purified DNA from the strains indicated according to the method described elsewhere (Vincart *et al.*, 2007). The detection rate of this assay has been determined to be 90, 100 and 100% at 10 log₁₀(copies ml⁻¹) for *B. pertussis* strains ATCC 9797, ATCC 9340 and Bord 201, respectively. Therefore, 10 log₁₀(copies ml⁻¹) was chosen as the detection limit for all strains evaluated in this report.

	Forward primer	Probe	Reverse primer
<i>B. pertussis</i>	TGCCGACTGGAACAACCA	GTCCATCGTCAAGACCGGTGAGCGCCAGCATGGCAT	CCATATCCAGGGCTCCGAC
BB variant 1	C-----A-----G-----C-----C-----C-----A-----CAAAG---T		
BB variant 2	-----A-----		
BB variant 3	-----C-----A-----		

Fig. 1. Alignment of the *B. pertussis* gene comprising covering the pertactin RT-PCR primers and probe with those of the three *B. bronchiseptica* (BB) sequence variants. Dashes represent conserved bases; substitutions are indicated by the appropriate letter. Although not depicted here, the corresponding sequences of the four currently available strains of *B. parapertussis* are identical to that of *B. bronchiseptica* variant 1.

RESULTS AND DISCUSSION

The earlier study that evaluated the performance characteristics of the pertactin RT-PCR included a single, unnamed isolate each of *B. bronchiseptica* and *Bordetella parapertussis* (Vincart *et al.*, 2007). A search of the GenBank database identified five *B. bronchiseptica* isolates and four *B. parapertussis* isolates for which the pertactin gene sequence corresponding to the RT-PCR probe and primers is available. We additionally determined the corresponding sequence from 83 strains of *B. bronchiseptica* selected from the National Animal Disease Center collection, representing isolates from humans and a variety of other host species. Alignment of the DNA sequences from the 88 *B. bronchiseptica* isolates revealed three unique variants, none of which is identical to the sequence reported for *B. pertussis* (Fig. 1). Variant 1 was found with the highest frequency in the *B. bronchiseptica* strains examined (64.8 %) and in all but one host species of origin (Table 1), as well as in the four *B. parapertussis* strains evaluated (GenBank accession nos BD409974, AX418063, X54547 and BX640426). One or more base mismatches are evident with respect to both the forward and reverse RT-PCR primers as well as the probe sequence proposed for identification of *B. pertussis*. Variants 2 and 3 were found in 22.7 and 12.5 % of *B. bronchiseptica* isolates, respectively, with the majority detected in strains of human or avian origin. Variants 2 and 3 contain only one or two base mismatches compared to the corresponding *B. pertussis* sequence.

These data suggest that some strains of *B. bronchiseptica* (specifically, those with sequence variants 2 or 3) may be misidentified as *B. pertussis* using the proposed pertactin RT-PCR (Vincart *et al.*, 2007). To address this possibility, the assay was carried out using purified DNA from *B. pertussis* and from two representatives each of *B.*

bronchiseptica sequence variants 1, 2 and 3. As shown in Table 2, *B. bronchiseptica* strains St Louis (variant 2), MBORD839 (variant 2), MO211 (variant 3) and F4563 (variant 3) were falsely identified as *B. pertussis* by the proposed pertactin RT-PCR assay. As expected, *B. bronchiseptica* strains RB50 and KM22 (both variant 1) were not identified as *B. pertussis*.

It has been suggested that the pertactin RT-PCR alleviates the problem of cross-reactivity with *Bordetella* species other than *pertussis* and may not require a confirmatory test (Vincart *et al.*, 2007). The specificity of the assay is purported to be superior to that of PCRs that target the repetitive insertion sequence IS481. However, these conclusions appear to have been based on the analysis of only a single strain from each of the other *Bordetella* species tested. Our results indicate that the pertactin RT-PCR is likely to incorrectly identify a large proportion of human *B. bronchiseptica* isolates as *B. pertussis*, since 43.5 % of the human isolates examined here possess sequence demonstrated to return a false-positive result. In fact, IS481-based assays may provide a specificity superior to that of the pertactin RT-PCR, since the insertion element has so far not been detected in any human *B. bronchiseptica* isolate (Diavatopoulos *et al.*, 2005; Register & Sanden, 2006; van der Zee *et al.*, 1997).

Other investigators have recently analysed the region of the pertactin gene that encodes the extracellular domain from 50 *B. bronchiseptica* isolates different from those in the present study (Diavatopoulos *et al.*, 2006). Those sequences overlap the region evaluated here, but do not include the 4 bp at the extreme 5' end. All but a single sequence, obtained from a seal isolate, match those reported here for variant 1, 2 or 3. The unique sequence in the seal isolate is identical to that of variant 1, except for an additional base

Table 1. Distribution of *B. bronchiseptica* pertactin RT-PCR sequence variants among host species of origin

Sequence variant	Host species											
	Dog	Human	Turkey/avian	Pig	Horse	Guinea pig	Rabbit	Seal	Sea otter	Koala	Leopard	Unknown
1	25	13	0	8	1	2	1	1	1	1	1	3
2	1	4	15	0	0	0	0	0	0	0	0	0
3	0	6	3	1	1	0	0	0	0	0	0	0

Table 2. Pertactin RT-PCR results using representative *B. bronchiseptica* isolates

Strain*	Ct value (cycle)
<i>B. pertussis</i> Tohama I	22.73
<i>B. bronchiseptica</i> RB50 (variant 1)	Undetermined
<i>B. bronchiseptica</i> KM22 (variant 1)	Undetermined
<i>B. bronchiseptica</i> St Louis (variant 2)	19.90
<i>B. bronchiseptica</i> MBORD839 (variant 2)	19.21
<i>B. bronchiseptica</i> MO211 (variant 3)	26.38
<i>B. bronchiseptica</i> F4563 (variant 3)	22.59

*All strains were evaluated using $10 \log_{10}(\text{copies ml}^{-1})$.

mismatch relative to *B. pertussis* in the region comprising the RT-PCR probe. Based on our RT-PCR results with variant 1 *B. bronchiseptica* strains, isolates with the novel sequence are not likely to be mistakenly identified as *B. pertussis*, although we have not directly tested this assumption. Due to the absence of sequence data for the bases corresponding to the extreme 5' end of the forward pertactin RT-PCR primer, it is not possible to conclude with certainty whether additional variants might exist among the remaining 49 *B. bronchiseptica* strains examined elsewhere (Diavatopoulos *et al.*, 2006).

Given the lack of specificity demonstrated by the results reported here, the proposed pertactin RT-PCR cannot be recommended for identification of *B. pertussis* in the absence of additional confirmatory testing. Further efforts are required to identify highly sensitive and specific targets suitable for definitive identification of *B. pertussis* by PCR.

ACKNOWLEDGEMENTS

The authors thank Michael Mullins and Gwen Nordholm for expert technical assistance.

REFERENCES

- Diavatopoulos, D. A., Cummings, C. A., Schouls, L. M., Brinig, M. M., Relman, D. A. & Mooi, F. R. (2005). *Bordetella pertussis*, the causative agent of whooping cough, evolved from a distinct, human-associated lineage of *B. bronchiseptica*. *PLoS Pathog* **1**, e45.
- Diavatopoulos, D. A., Hijnen, M. & Mooi, F. R. (2006). Adaptive evolution of the *Bordetella* autotransporter pertactin. *J Evol Biol* **19**, 1931–1938.
- Register, K. B. (2004). Comparative sequence analysis of *Bordetella bronchiseptica* pertactin gene (*prn*) repeat region variants in swine vaccines and field isolates. *Vaccine* **23**, 48–57.
- Register, K. B. & Sanden, G. N. (2006). Prevalence and sequence variants of IS481 in *Bordetella bronchiseptica*: implications for IS481-based detection of *Bordetella pertussis*. *J Clin Microbiol* **44**, 4577–4583.
- Register, K. B., Boisvert, A. & Ackermann, M. R. (1997). Use of ribotyping to distinguish *Bordetella bronchiseptica* isolates. *Int J Syst Bacteriol* **47**, 678–683.
- Riffelmann, M., Wirsing von Konig, C. H., Caro, V. & Guiso, N. (2005). Nucleic acid amplification tests for diagnosis of *Bordetella* infections. *J Clin Microbiol* **43**, 4925–4929.
- van der Zee, A., Mooi, F., Van Embden, J. & Musser, J. (1997). Molecular evolution and host adaptation of *Bordetella* spp.: phylogenetic analysis using multilocus enzyme electrophoresis and typing with three insertion sequences. *J Bacteriol* **179**, 6609–6617.
- Vincart, B., De Mendonca, R., Rottiers, S., Vermeulen, F., Struelens, M. J. & Denis, O. (2007). A specific real-time PCR assay for the detection of *Bordetella pertussis*. *J Med Microbiol* **56**, 918–920.