Prevalence and Sequence Variants of IS481 in *Bordetella bronchiseptica*: Implications for IS481-Based Detection of *Bordetella pertussis*

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Received 23 June 2006/Returned for modification 10 August 2006/Accepted 11 October 2006

We report the prevalence in *Bordetella bronchiseptica* of IS481, a frequent target for diagnosis of *Bordetella pertussis*, as approximately 5%. However, PCR amplicons of the predicted size were detectable in 78% of IS481-negative strains. Our results suggest that PCR targeting IS481 may not be sufficiently specific for reliable identification of *B. pertussis*.

*Bordetella pertussis* is the etiologic agent of whooping cough, an acute respiratory disease occurring exclusively in humans. Widespread vaccination provides a high level of protection in children, but unvaccinated populations and cohorts in whom vaccination-induced immunity has waned remain at risk. Recently, a reemergence of the disease within highly vaccinated populations has occurred, and transmission from adult reservoirs to unimmunized or partially immunized children is of increasing concern in many areas of the world (36). A sensitive and specific method for identification of *B. pertussis* is crucial for monitoring prevalence, accurately defining epidemiology, and optimizing intervention and prevention strategies, including management of outbreaks. Culture remains the diagnostic standard but suffers from poor sensitivity, slow turnaround time, and practical difficulties. Additionally, it is necessary to distinguish between *B. pertussis* and *Bordetella parapertussis*, which may cause a milder pertussis-like syndrome in humans. PCR assays that differentiate between these bacteria, either alone or combined with traditional culture, are increasingly being implemented in diagnostic laboratories.

A frequently used target for PCR detection of *B. pertussis* is the repetitive element IS481 (29, 30), which is absent in *B. parapertussis* (8, 48) but found in approximately 50 to a few hundred copies in *B. pertussis* (16, 27, 34, 35, 42). While IS481 is generally regarded as specific for *B. pertussis*, few studies definitively examine the prevalence of IS481 in the closely related agent *Bordetella bronchiseptica*. Despite recent evidence that *B. pertussis* may have evolved from a human-associated lineage of *B. bronchiseptica* (8), the latter agent is primarily associated with disease in mammals other than humans and, therefore, has been considered to have little clinical significance. However, human illness and carriage associated with *B. bronchiseptica* is on the rise, particularly in infants or immunocompromised hosts with exposure to carrier animals (1, 4, 5, 6, 11, 14, 15, 17, 26, 31, 32, 38, 44, 52, 54, 55). While rare, disease in immunocompetent adults may also occur (5, 22, 26, 54).

Analysis of a few isolates by Southern blotting suggested that IS481 may not be found in *B. bronchiseptica* (19, 27, 34), but one study provided evidence for a single copy in an isolate of unstated origin (16). van der Zee et al. (50) reported a prevalence of approximately 1% based on a diverse group of 144 strains. Unfortunately, the data upon which this conclusion is based were not presented, and specific methods used to identify IS481 are not detailed either in the original report or in the supporting references provided (49, 51). A more recent investigation evaluating 91 *B. bronchiseptica* isolates from a variety of host species identified only 2 (both equine) positive by PCR for an amplicon derived from the central region of IS481 (8). From a clinical perspective, it is important to know whether amplicons can be generated from *B. bronchiseptica* strains using IS481-specific primers designed to detect *B. pertussis*, potentially leading to an erroneous diagnosis. A review of the literature encompassing PCR methods used to identify *B. pertussis* reveals that “diagnostic” amplicons were obtained from *B. bronchiseptica* isolates in at least five investigations using primers specific for the 5' end of the repetitive element (7, 13, 16, 20, 21) but by none using primers that target the 3' end (3, 9, 10, 45). No diagnostic PCRs targeting the central region of IS481 have been proposed. Only about 20 *B. bronchiseptica* isolates were evaluated overall in these studies (the exact number being unclear from the details provided), and only a few were obtained from humans. The goal of the present study was to establish the prevalence of IS481 based on a larger number of *B. bronchiseptica* isolates, including those of human origin, and to assess the potential for misidentification of IS481-containing strains of *B. bronchiseptica* as *B. pertussis* when using IS481-specific PCR primers.

PCR primers IS481-1 and IS481-2 (Fig. 1) were used to screen for IS481 in *B. bronchiseptica*. This primer set is used by the Pertussis Program Laboratory of the Centers for Disease Control and Prevention for IS481-based identification of *B. pertussis* and encompasses a 252-bp region of the insertion sequence frequently selected as a target in *B. pertussis* diag-

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† Supplemental material for this article may be found at http://jcm.asm.org/.
‡ Published ahead of print on 25 October 2006.
FIG. 1 continues
nmonic PCRs (3, 9, 10, 45, 53). Reaction mixtures included 0.4 μM each primer, 1 U AmpliTaq polymerase (Applied Biosystems, Foster City, CA), 2.5 μl 10X buffer II (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 2.5 mM MgCl₂, 200 μM deoxynucleotide triphosphates, and either 100 ng of purified DNA (\(17\)), 0.5 μl of a cell lysate (\(123\)), in a final volume of 25 μl. PCR was carried out in an Applied Biosystems (Foster City, CA) 9700 thermal cycler. Cycling conditions were as follows: 3 min at 95°C; 35 cycles, with 1 cycle consisting of 95°C for 15 s, 58°C for 30 s, and 72°C for 30 s; and a final extension step of 72°C for 10 min. A total of 149 \(B. bronchiseptica\) isolates were evaluated (see Table S1 in the supplemental material). These were obtained primarily from humans and companion or farm animal species; some have previously been examined for IS\(481\) by other methods (8, 50). \(B. pertussis\) (Tohama) was used as a positive control. The integrity of templates and reagents was established by parallel testing with a 16S rRNA-specific PCR (41). Five microliters of each PCR mixture was analyzed by agarose gel electrophoresis.

Unexpectedly, an amplicon of the predicted size was detected from 118 \(B. bronchiseptica\) isolates, including 22 of 24 isolates of human origin, following PCR with primer pair IS\(481\)-1/IS\(481\)-2. Strain RB50, included as one of the negative controls since it does not contain IS\(481\) (35), gave false-positive results. One or two additional bands were present in reactions from 12 isolates. No amplification products were visualized from negative controls containing water in place of template, included with every set of PCRs. Representative results are shown in Fig. 2A and B. Increasing the annealing temperature to 60°C (primer melting temperature is 62 to 63°C) reduced the intensities of some weakly fluorescent amplicons but did not completely eliminate them. Significant differences in band intensity apparent in reaction mixtures with a standardized amount of purified DNA (Fig. 2A) suggest that some isolates may contain one or more copies of IS\(481\), although it seems likely that weaker amplicons of the expected size represent false-positive results.

The strongly fluorescent amplicons of ∼250 bp generated with purified DNA from three \(B. bronchiseptica\) isolates, representing three different species of origin, were each purified with spin columns (QIAGEN, Valencia, CA) and sequenced directly (minimum of three reactions, at least one from each strand) as described previously (39). Sequence alignment showed 98.8 to 100% identity with the sequence originally reported for this region of \(B. pertussis\) IS\(481\) (27; K. B. Regis-Lampalter et al. (27)).

![FIG. 1—continued](image-url)
seven isolates (Table 1; see Table S1 in the supplemental material), all of which displayed strongly fluorescent amplicons of this size in ethidium bromide-stained gels. Representative results are shown in Fig. 2C.

The 7 confirmed IS481-positive isolates, as well as 48 PCR-positive isolates whose amplicons failed to hybridize with the IS481 probe, were further evaluated by Southern blotting with genomic DNA. Following digestion of 3 μg of purified DNA with PstI, for which no recognition sites are present in B. pertussis IS481 (27, 28), fragments were separated on 0.6% agarose gels, and blots were prepared and hybridized with the B. bronchiseptica IS481 probe as described above. Signals were detected only from the seven isolates already identified as containing IS481, and at least some appeared to contain more than a single copy (K. B. Register, unpublished data). Additional Southern blot analysis using alternative restriction enzymes clearly demonstrated all seven isolates possess multiple copies of IS481. Results obtained with NarI, which has the most frequently occurring recognition sequence of the enzymes tested, are shown in Fig. 3.

These data suggest that moderately to weakly fluorescent IS481-1/IS481-2 amplicons, although of the predicted size, are not derived from IS481. Amplicons obtained from six IS481-negative B. bronchiseptica isolates, including RB50, were sequenced in an attempt to determine their origin. All sequences shared 100% identity with one another as well as with the 5′ and flanking region of an RB50 open reading frame predicted to encode an extracellular solute-binding protein (locus tag BB5004), identified from a BLASTN search of the genome database (http://www.sanger.ac.uk/Projects/B_bronchiseptica). Alignment of the IS481 primers with this segment of the RB50 genome revealed likely primer-binding locations which would result in a product of 252 bp (Fig. 4), consistent with our observations. No other regions of significant identity with the IS481-1/IS481-2 amplicon were noted within the sequence of

![FIG. 2. Amplicons from PCRs with IS481-1/IS481-2 primers and B. bronchiseptica purified DNA (A) or cell lysates (B) detected by ethidium bromide staining. (A) Lanes: 1, rabbit isolate (M116919/02/1); 2, dog isolate (MBORD595); 3, guinea pig isolate (MBORD673); 4, cat isolate (MBORD733); 5, human isolate (W48661); 6, turkey isolate (4448); 7, rabbit isolate (RB50); 8, negative control; 9, dog isolate (MBORD599); 10, B. pertussis. Relative positions of DNA size markers are indicated to the left of the gel. (B) Lanes: 1, DNA size markers; 2, B. pertussis; 3 and 4, guinea pig isolates (MBORD762 and MBORD665, respectively); 5, dog isolate (MBORD839); 6, pig isolate (5238); 7, human isolate (MO289); 8, rabbit isolate (MBORD835); 9, pig isolate (SG8); 10 and 11, rabbit isolates (RB50 and M602871.001, respectively); 12, negative control. (C) Southern blot of the gel in panel B hybridized with an IS481-specific probe as described in the text.](image)

![FIG. 3. Southern blots with NarI-digested B. bronchiseptica genomic DNA or, for comparison, B. pertussis genomic DNA hybridized to an IS481-specific probe. (A) Lanes: 1, dog isolate (MBORD595), 2 to 6, guinea pig isolates (MBORD665, MBORD666, MBORD668, MBORD669, and MBORD678, respectively); 7, horse isolate (MBORD731). (B) Lanes: 1, B. pertussis; 2, B. bronchiseptica RB50. Relative positions of DNA size markers are indicated to the left (A) or right (B) of the gel.](image)

<table>
<thead>
<tr>
<th>Host species</th>
<th>IS481 positive</th>
<th>IS481 negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>0</td>
<td>2 (22)</td>
</tr>
<tr>
<td>Dog</td>
<td>1</td>
<td>1 (28)</td>
</tr>
<tr>
<td>Cat</td>
<td>0</td>
<td>0 (8)</td>
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<tr>
<td>Horse</td>
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<td>0 (6)</td>
</tr>
<tr>
<td>Rabbit</td>
<td>0</td>
<td>3 (17)</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>5</td>
<td>1 (5)</td>
</tr>
<tr>
<td>Pig</td>
<td>0</td>
<td>10 (17)</td>
</tr>
<tr>
<td>Turkey</td>
<td>0</td>
<td>2 (11)</td>
</tr>
<tr>
<td>Seal</td>
<td>0</td>
<td>3 (2)</td>
</tr>
<tr>
<td>Sea otter</td>
<td>0</td>
<td>1 (0)</td>
</tr>
<tr>
<td>Leopard</td>
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<td>0 (1)</td>
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<tr>
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<td>1 (1)</td>
</tr>
<tr>
<td>Total</td>
<td>7</td>
<td>24 (118)</td>
</tr>
</tbody>
</table>

* Numbers of isolates in which an amplicon of the predicted size could be detected in agarose gels and which hybridized with an IS481-specific probe.

* Numbers of isolates from which no amplicon could be detected in agarose gels.

* The numbers in parentheses are the numbers of isolates for which an amplicon of the predicted size that failed to hybridize with an IS481-specific probe was present (false positives).
the extracellular solute-binding protein gene spanned by the primers.

Because of this unexpected finding, additional analysis was carried out to assess the potential for unintended primer binding to DNA from other genera that might contaminate clinical samples or be inadvertently introduced from the environment. A BLAST search of the GenBank RefSeq database revealed regions of 100% identity with the 13 to 15 consecutive 3′-terminal bases of IS481-1 in sequences derived from *Aspergillus oryzae*, *Candida alibicans*, and *Streptomyces avermitilis* and of IS481-2 in sequences from *Saccharomyces cerevisiae*, *Homo sapiens*, and multiple strains of both *Streptococcus pyogenes* and *Escherichia coli*. However, when the database was queried with a single sequence comprised of the concatenated primers and an N22 spacer, no alignments with the potential to result in a single sequence comprised of the concatenated primers and a N22 spacer, no alignments with the potential to result in amplification of a product were identified. These results suggest that while there may be sequences in human DNA and some bacterial and fungal species capable of weakly binding IS481-1 or IS481-2, a PCR amplicon is not likely to be generated.

Since some *B. bronchiseptica* strains possess at least a portion of IS481, misidentification of such strains as *B. pertussis* could occur when IS481-based PCR assays are used as the basis for a diagnosis of whooping cough. To more precisely evaluate the potential for misidentification with particular primer sets, we determined the DNA sequence of the *B. bronchiseptica* insertion element. Primers specific for the 5′ and 3′ ends of *B. pertussis* Tohama IS481 (IS481-5 and IS481-12 [Fig. 1]) were used to generate amplicons comprising the entire insertion element from the seven IS481-containing *B. bronchiseptica* isolates. Amplicons were purified and directly sequenced as described above. Sequence alignment revealed two variants of IS481, neither of which is identical to any of the three variants reported for *B. pertussis* (Fig. 1). It should be noted that, since all positive isolates have multiple copies of IS481 and the DNA sequences were obtained from purified PCR products, it is not possible to rule out the existence of additional, less frequently occurring variants. *B. bronchiseptica* IS481 variant 1 was found only in isolate MBORD731 and shares the greatest degree of identity with *B. pertussis* variants (one to three base substitutions depending on the variant used for comparison). The IS481 sequences from the six remaining *B. bronchiseptica* isolates are identical to one another (*B. bronchiseptica* IS481 variant 2) and have 25 to 27 base substitutions compared to the *B. pertussis* variants. No base substitutions are shared between *B. pertussis* IS481 variants and those of *B. bronchiseptica*. Interestingly, one substitution in *B. bronchiseptica* IS481 variant 2 eliminates a purported start codon for ORF3 (28), suggesting the insertion sequence may not be functional in most IS481-containing *B. bronchiseptica* isolates and perhaps explaining both its low prevalence and copy number. The single *B. bronchiseptica* isolate with an intact ORF3 start codon contains many more copies of IS481 than the other positive isolates. Alternatively, if IS481 had been acquired relatively recently, insufficient time may have elapsed for generating additional copies.

On the basis of a comparison of the *B. bronchiseptica* IS481 sequences with those of IS481 primers used for conventional PCR identification of *B. pertussis* (2, 3, 9, 10, 12, 13, 16, 18, 20, 21, 23, 25, 37, 45, 46, 47, 53), it seems likely that misidentification of IS481-containing strains of *B. bronchiseptica* would occur. Many of the suggested primers are 100% identical to the *B. bronchiseptica* sequence reported here, while others have a few base substitutions which may still permit amplification. A subset of the most commonly used primer pairs, including IS481-5/IS481-6 (18, 23, 46, 47), IS481-7/IS481-8 (21), IS481-5/IS481-8 (25, 29), IS481-5/IS481-10 (13, 16, 19), IS481-9/IS481-12 (9), IS481-11/IS481-14 (53), and IS481-13/IS481-16 (45; see Fig. 1 for primer sequences and locations), was tested with genomic DNA from the seven *B. bronchiseptica* isolates containing IS481. Selection of an appropriate melting temperature during cycling was problematic, since the temperature used by different investigators for the same primer pair sometimes varied greatly (e.g., a range of 55°C to 66°C for IS481-5/ IS481-6) and many primer pairs are poorly matched (some differing by 10°C or more). In keeping with the conditions reported most frequently, primer pairs were tested at both 55°C and 58°C. PCR components and other cycling parameters were as described above. Amplicons were obtained from all isolates at both melting temperatures with all primer pairs tested, including those having one or more mismatched bases in comparison with *B. bronchiseptica* IS481 sequence. Representative results are shown in Fig. 5. Although no false-positive results were noted with strain RB50, faint bands ~100 to 150 bp larger and/or smaller than the IS481-specific amplicons were evident for a few of the *B. bronchiseptica* isolates using two of the three primer pairs directed against the 3′ portion of the insertion element (K. B. Register, unpublished data).

Our data indicate an IS481 prevalence of roughly 5% in the group of *B. bronchiseptica* isolates examined (Table 1); others have reported a prevalence of roughly 1 to 2% (8, 50). The isolates evaluated by Diavatopoulos et al. (8) include 35 also evaluated here. Our results are concordant with the exception of strain MBORD669, previously reported to be IS481 negative but found here to be positive. Comparison of the sequence of MBORD669 IS481 with the primers used by Diavatopoulos et al. reveals the sequences are identical except for a single, internal base mismatch in the reverse primer, which seems unlikely to explain the discrepancy. Accidental cross-contamination of cultures in our laboratory also seems unlikely since
with the IS481 insertions. Detection of many identically sized restriction fragments in a laboratory could perhaps assist in resolving the discrepancy. Comparative evaluation of MBORD669 DNA obtained from each of the positive isolates (40; K. B. Register, unpublished data). A comparator of the probe in all strains positive by Southern blotting and cross-hybridization of the probe with IS481 could be postulated that our results are confounded by the presence of IS1002, an insertion sequence closely related to IS481. However, the degree of sequence identity between IS1002 (49) and our IS481 probe is insufficient to withstand the stringency of the conditions used for hybridizations. Detection of many identically sized restriction fragments with the IS481 probe in all strains positive by Southern blotting (Fig. 3A; K. B. Register, unpublished data) also argues against cross-hybridization of the probe with IS1002.

The prevalence and sequences of IS481 in B. bronchiseptica reported here suggest that the specificity of this target for identification of B. pertussis is less than presently perceived. Although the insertion sequence was not detected in any of the 26 human isolates tested, its occurrence in strains obtained from farm and companion animals suggests that transmission of IS481-containing B. bronchiseptica strains to humans remains a possibility. Also of concern is the finding that secondary amplicons not derived from IS481 but of the predicted size, as well as some with slightly different mobilities, were often apparent when using some primers derived from the 3′ region of B. pertussis IS481. Utilization of amplicon-specific probes in real-time PCR formats may alleviate this problem, but careful evaluation of IS481-based diagnostic assays for B. pertussis is essential, including an analysis of proven IS481-positive and -negative strains of B. bronchiseptica. It is unclear whether B. bronchiseptica isolates reported as PCR positive in assays targeting the 5′ region of IS481 are, in fact, IS481 positive (13, 16, 20, 21). The details available suggest that at least some amplicons may have been derived from secondary primer-binding sites (16, 21) as observed for most isolates in this study with primers IS481-1/IS481-2.

Our observations, together with reports identifying numerous IS481-positive Bordetella holmesii strains (21, 24, 45, 46, 47), raise significant concerns as to the likelihood of a false diagnosis of B. pertussis if IS481 PCR is the only test carried out, as observed in a recent quality assessment proficiency panel (33). All laboratories utilizing IS481 PCR mistakenly identified both B. bronchiseptica and B. holmesii as B. pertussis. The suggestion that IS481 PCR assays are sufficiently specific for diagnosis of B. pertussis (43) may be premature. Consequently, promising alternative and/or confirmatory PCR targets should continue to be evaluated, and clinical sensitivity and specificity of IS481-based PCR for pertussis need to be more rigorously defined.

Nucleotide sequence accession numbers. The GenBank accession numbers for the DNA sequences determined in this study are EF043395 to EF043401.

We gratefully acknowledge the technical assistance of Michael Mulins. We are indebted to David Alt and the National Animal Disease Center Genomics Unit for DNA sequence data and to B. Rath, G. Foster, R. Welsh, and H.-J. Rusing for providing B. bronchiseptica isolates.

REFERENCES


FIG. 5. Amplicons obtained from PCR with primers targeting the 5′ end (IS481-5/IS481-6 [A]) or 3′ end (IS481-13/IS481-16 [B]) of B. pertussis IS481 using B. bronchiseptica genomic DNA and a melting temperature of 58°C. Lanes: 1, DNA size markers; 2, MBORD595; 3, MBORD665; 4, MBORD666; 5, MBORD668; 6, MBORD669; 7, MBORD678; 8, MBORD731; 9, B. pertussis; 10, RB50. Relative positions of DNA size markers are indicated to the left of the gels.