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A Simplified Sequence-Based Identification Scheme for *Bordetella* Reveals Several Putative Novel Species

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The differentiation of *Bordetella* species, particularly those causing human infection, is problematic. We found that sequence analysis of an internal fragment of *nrdA* allowed differentiation of the currently named *Bordetella* species. Analysis of 107 "*Bordetella*" isolates recovered almost exclusively from human respiratory tract specimens identified several putative novel species.

The genus *Bordetella* currently consists of eight species: *Bordetella avium*, *Bordetella bronchiseptica*, *Bordetella hinzii*, *Bordetella holmesii*, *Bordetella parapertussis*, *Bordetella pertussis*, *Bordetella petrii*, and *Bordetella trematum*. Another species, "*Bordetella ansorpii*," has been proposed, but not validly named, and is represented by only a couple of reported isolates (1). Historically, the need to reliably detect and differentiate *Bordetella* species in clinical samples has been limited by the predominant role that *B. pertussis* was believed to play in human infection. However, other *Bordetella* species appear to be involved in human infection more commonly than previously appreciated. *B. parapertussis*, in particular, likely accounts for a considerable minority of cases of whooping cough (pertussis) (2) and, recently, *B. holmesii* was identified in a large community outbreak of a pertussis-like illness in Ohio (3). Currently, the most common diagnostic testing involves various real-time PCR (RT-PCR) assays targeting pertussis toxin or toxin promoter genes and/or variable number insertion elements to detect and differentiate *Bordetella* species directly in clinical specimens. These assays, together with combinations of growth properties, biochemical tests, serotyping, and sequencing of a portion of the 16S rRNA gene, are used to differentiate *Bordetella* species recovered in culture. However, these methods are cumbersome and specific interpretive criteria for species differentiation are lacking (4). We recently showed that sequence analysis of *nrdA*, which encodes the ribonucleoside-diphosphate reductase alpha chain, can reliably differentiate species within the genus *Achromobacter* (5, 6). Given the phylogenetic relatedness of *Bordetella* and *Achromobacter*, we sought to determine if sequence analysis of this locus would similarly allow reliable differentiation of cultured isolates of *Bordetella* species, thereby providing the basis for further analysis of this target for direct detection of *Bordetella* in clinical specimens.

The 165 strains analyzed in this study are listed in Table S1 in the supplemental material. A total of 107 isolates recovered from culture of biologic specimens primarily from persons with and without cystic fibrosis (CF) were referred to the *Burkholderia cepacia* Research Laboratory and Repository (University of Michigan) from 35 clinical microbiology laboratories in 23 states in the United States between 2001 and 2013. DNA preparations from 30 *Bordetella* strains cultured primarily from human nasopharyngeal swabs were obtained from Nationwide Children's Hospital (Columbus, Ohio). These 30 strains had been identified as *B. pertussis*, *B. parapertussis*, or *B holmesii* by traditional and molecular methods (3). Sixteen type and taxonomic reference strains were obtained from the BCCM/LMG culture collection (Ghent, Belgium). DNA sequences from the 12 remaining *Bordetella* strains were obtained from whole-genome sequences available at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/genome/browse/).

All bacterial cultures were incubated aerobically at 32°C for 24 h on Mueller-Hinton agar. DNA was prepared from single colonies as described previously (5). PCR primers P1 and P2, with the following 5′ → 3′ nucleotide sequences, were manually designed to target *nrdA*: P1, GCCCCGACCTGCACGAC; P2, CCGGASAG RTTCGATTTGACGTAC. DNA amplification and sequencing were performed as previously described with the following modification (5). After an initial denaturation for 2 min at 95°C, 30 cycles, with each consisting of 30 s at 94°C, 30 s at 58°C, and 60 s at 72°C, were completed. A final extension of 5 min at 72°C was applied with an infinite hold at 8°C. The bacterial 16S rRNA gene was amplified by PCR as previously described (7). DNA sequencing and editing of sequence chromatograms was performed as described previously (5). *nrdA* sequences were trimmed to 765 bp and aligned using MegAlign (DNAStar, Madison, WI, USA) with Clustal W to generate dendrograms with 1,000 bootstrap replications using the MegAlign default parameters. For validation of distinctness of sequence similarity clusters, the *k* parameter was calculated as previously described (8).

All *nrdA* sequences generated in this study can be found at the PubMLST site at http://pubmlst.org/bordetella (9).

A dendrogram based on the 765-bp fragment of the *nrdA* gene from 12 *Bordetella* strains for which whole-genome sequences

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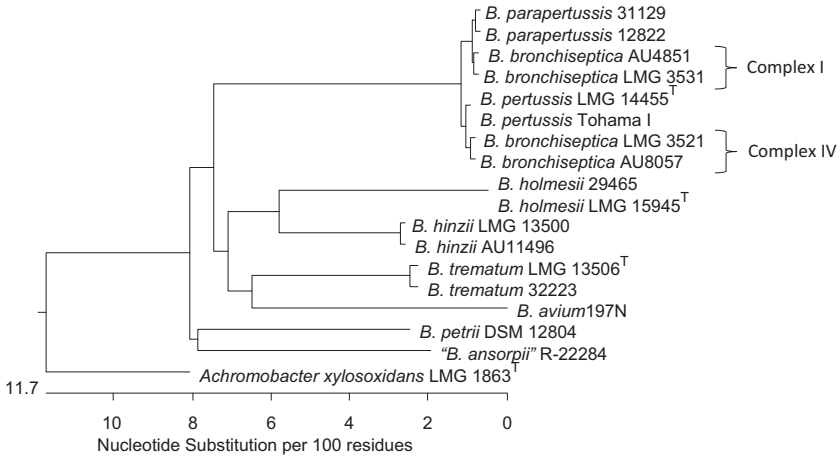

FIG 1 *nrdA*-based dendrogram containing two (if available) representative strains of the nine named species in the genus *Bordetella*. *B. bronchiseptica* complex I and *B. bronchiseptica* complex IV are represented (10). The tree is rooted with *Achromobacter xylosoxidans*.

were available from NCBI, together with sequences from 16 taxonomic reference strains and 30 *Bordetella* strains previously identified to the species level (3), separated strains into clusters representing each of the named species in the genus (Fig. 1; see Fig. S1 in the supplemental material). As expected, *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* showed greater similarity at this locus than the other species in the genus. Two distinct clusters of *B. bronchiseptica* were apparent, in agreement with the separation of this species into two groups (complexes I and IV), described by Diavatopoulos and colleagues (10), based on sequence analysis of seven housekeeping genes.

When the 107 clinical isolates were added to the 58 reference strains described above, the *nrdA* sequence-based dendrogram again showed clustering corresponding to the named species in the genus (see Fig. S2 in the supplemental material). However, several strains grouped into new clusters apart from those representing the named *Bordetella* species. Several individual strains did not cluster with any others but rather occupied distinct branches in the tree. Nine clusters were each comprised of at least two strains with different *nrdA* sequences, allowing for calculation of a *k* parameter to discern how distinct these were from each other and from the nine named *Bordetella* species. The *k* parameters for these nine clusters ranged from 3.88 to 167.43 (Table 1), indicating that each of these indeed represented a distinct genogroup, designated *Bordetella* genogroups 1 through 9. Another cluster consisted of two strains with the same *nrdA* sequence, precluding calculation of a *k* parameter. Nevertheless, for the sake of description, this cluster was designated genogroup 10. The six strains occupying solitary branches were designated genogroups 11 to 16.

TABLE 1 *k* parameters for novel *Bordetella* genogroups

Reference group (no. of strains available)	Closest neighbor	Intragroup divergence	Intergroup divergence	*k*
B. genogroup 1 (5)	*B.* genogroup 5	0.46	8.64	18.78
B. genogroup 2 (7)	*B. petrii*	0.06	5.93	98.83
B. genogroup 3 (8)	*B.* genogroup 10	0.07	9.44	134.86
B. genogroup 4 (3)	*B.* genogroup 7	0.07	11.72	167.43
B. genogroup 5 (2)	*B.* genogroup 1	0.10	8.64	86.40
B. genogroup 6 (8)	*B. bronchiseptica* I	0.29	2.63	9.07
B. genogroup 7 (3)	*B.* genogroup 4	0.07	11.67	166.71
B. genogroup 8 (2)	*B.* genogroup 14	1.30	5.05	3.88
B. genogroup 9 (2)	*B.* genogroup 3	0.30	10.24	34.13

A dendrogram based on 16S rRNA gene sequences was constructed to include one strain from each named *Bordetella* species, including *B. bronchiseptica* complex I and *B. bronchiseptica* complex IV, as well as one representative from each of the 16 novel genogroups (see Fig. S3 in the supplemental material). As expected, *B. parapertussis*, *B. bronchiseptica* complex I, and *B. bronchiseptica* complex IV could not be distinguished from each other at this locus. Of note, *nrdA* genogroup 6 was also indistinguishable from *B. parapertussis* and *B. bronchiseptica* by 16S rRNA gene sequence analysis.

Among the several species of human and animal pathogens in the genus *Bordetella*, *B. pertussis* continues to garner the most attention in human health, particularly with the increased incidence of pertussis in the United States in recent years despite the routine use of an effective vaccine (11). *B. parapertussis* and *B. holmesii* can also cause a pertussis-like illness that may be more common than generally appreciated (2). Recently, Rodgers and colleagues (3) reported a community outbreak of a pertussis-like illness in Ohio involving 918 persons. Among the nasopharyngeal swab samples available from 298 persons, *Bordetella* species were detected in 164 based on a positive IS*481* PCR assay. By the use of multiple PCR assays, 68% of these samples were confirmed as *B. pertussis*, 29% as *B. holmesii*, and 2% as containing both species.

The assessment of the prevalence of these species in human respiratory illness and a better understanding of the natural history of *Bordetella* infection and analyses of pertussis vaccine efficacy are predicated on reliable methods to detect and differentiate these species; however, this can be challenging. Currently, an RT-PCR assay targeting IS*481* is most often used to identify *B. pertussis*, which contains 50 to 238 copies of this insertion element, in clinical specimens (12, 13). However, IS*481*or IS*481*-like sequences can be found in *B. holmesii* and in some strains of *B. bronchiseptica* (14). Thus, without further differentiation, IS*481*-positive samples may be falsely reported as *B pertussis*. Additional PCR assays to detect and differentiate *Bordetella* species include

those targeting the insertion elements hIS*1001* (found in *B. holmesii* only) and pIS*1001* (found in *B. parapertussis* and infrequently in *B. bronchiseptica*) (15). These assays may be combined with PCR assays targeting the toxin gene *ptxS1* (detected in *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*) and/or intact *ptxA*-Pr sequences (detected in *B. pertussis*). Mutated *ptxA*-Pr sequences may be found in *B. parapertussis* and *B. bronchiseptica*. Despite the use of these assays, the molecular detection and differentiation of *Bordetella* species remain problematic (4, 16).

To develop a reliable streamlined assay to differentiate *Bordetella* species recovered in culture, we drew upon our recent experience (5, 6) using analysis of a single genomic locus to identify species in the genus *Achromobacter*, which is phylogenetically closely related to *Bordetella*. In that work, we found that DNA sequence analysis of a 765-bp segment of *nrdA* enabled differentiation of the seven named *Achromobacter* species that was comparable to the separation of these species provided by multilocus sequence analysis. Further, in an analysis of 147 strains, we identified 14 novel *Achromobacter* genogroups, several of which were found to cause infection in persons with CF (6). In subsequent and ongoing work, comprehensive taxonomic assessment has confirmed that these groups represent novel *Achromobacter* species. Eight of these species now have been formally described (17, 18).

In the present study, we found that *nrdA* sequence analysis differentiated the currently named *Bordetella* species (Fig. 1). This includes separation of *B. pertussis*, *B. parapertussis*, and the two *B. bronchiseptica* subpopulations (complexes I and IV) described by Diavatopoulos and colleagues (10). All *B. pertussis* strains were clonal at this locus, even though these were from geographically diverse locations (United States, Netherlands, China, and Japan), with some being initially recovered more than 60 years ago. All *B. parapertussis* strains also had identical sequences at this locus, as did the seven available *B. holmesii* isolates. In contrast, 12 alleles were found among the 63 *B. bronchiseptica* isolates, 3 alleles were found among the 11 *B. hinzii* isolates, and 3 were noted in the four *B. trematum* isolates included in the study.

In extending our analysis to a larger collection of *Bordetella* isolates cultured from human respiratory specimens, most of which were from persons with CF, we noted that several groups or individual strains appeared to cluster apart from the known *Bordetella* species on the *nrdA*-based dendrogram. To assess the distinctness of these groups, we calculated the *k* parameter, which is the ratio of the mean intergroup sequence divergence to the mean intragroup sequence divergence (8). A *k* parameter value greater than 2 indicates that a group is distinct from its closest neighbor. We found that genogroups 1 through 9, each of which included strains with two or more different *nrdA* sequences, would all be considered distinct based on their respective *k* parameters, indicating that each most likely represents a novel species. Analysis of a greater number of strains that cluster with isolates in genogroups 10 through 16 will be required to determine if these too are sufficiently genetically distinct to represent novel taxa.

In summary, we have shown that *nrdA* sequence analysis allows for the reliable differentiation of the currently named *Bordetella* species recovered in culture. In addition, we have identified several distinct genogroups among "*Bordetella*-like" isolates recovered from human respiratory specimens. Analysis of the 16S rRNA gene from representatives of each of these novel genogroups (see Fig. S3 in the supplemental material) suggests that some (genogroups 3, 8, 9, 10, 11, 14, and 15) may represent a novel genus, closely related to *Achromobacter* and *Bordetella*. Clarification of the taxonomy of these genogroups will provide the basis for further studies to assess the utility of *nrdA* amplification and sequence analysis for the direct detection and differentiation of *Bordetella* species in respiratory specimens.

Nucleotide sequence accession numbers. The 16S rRNA gene sequences generated in this study were deposited in GenBank (http://www.ncbi.nlm.nih.gov/GenBank) and are assigned the following accession numbers: EU082134, EU082135, EU082146, EU082151, EU082156, EU082157, EU082159, EU082162, and KF601902 through KF601915.

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