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Comparison of ribotyping and sequence-based typing for discriminating among isolates of *Bordetella bronchiseptica*

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ABSTRACT

*Pvu*II ribotyping and MLST are each highly discriminatory methods for genotyping *Bordetella bronchiseptica*, but a direct comparison between these approaches has not been undertaken. The goal of this study was to directly compare the discriminatory power of *Pvu*II ribotyping and MLST, using a single set of geographically and genetically diverse strains, and to determine whether subtyping based on repeat region sequences of the pertactin gene (*prn*) provides additional resolution. One hundred twenty-two isolates were analyzed, representing 11 mammalian or avian hosts, sourced from the United States, Europe, Israel and Australia. Thirty-two ribotype patterns were identified; one isolate could not be typed. In comparison, all isolates were typeable by MLST and a total of 30 sequence types was identified. An analysis based on Simpson's Index of Diversity (SID) revealed that ribotyping and MLST are nearly equally discriminatory, with SIDs of 0.920 for ribotyping and 0.919 for MLST. Nonetheless, for ten ribotypes and eight MLST sequence types, the alternative method discriminates among isolates that otherwise type identically. Pairing *prn* repeat region typing with ribotyping yielded 54 genotypes and increased the SID to 0.954. Repeat region typing combined with MLST resulted in 47 genotypes and an SID of 0.944. Given the technical and practical advantages of MLST over ribotyping, and the nominal difference in their SIDs, we conclude MLST is the preferred primary typing tool. We recommend the combination of MLST and *prn* repeat region typing as a high-resolution, objective and standardized approach valuable for investigating the population structure and epidemiology of *B. bronchiseptica*.

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1. Introduction

Bordetella bronchiseptica is a widespread bacterial pathogen that infects a variety of domesticated and wildlife animal species. It causes tracheobronchitis (kennel cough) in dogs, bronchopneumonia in numerous laboratory, companion and wild animals, neonatal pneumonia in piglets and is an important contributor to swine atrophic rhinitis and porcine respiratory disease complex (Brockmeier et al., 2012). Human infections occasionally occur, most frequently in immunocompromised individuals, but related illness in healthy adults and children has also been reported (Lo Re, et al., 2001; Llombart et al., 2006; Wernli et al., 2011).

Multilocus sequence typing (MLST), based on partial sequencing of seven housekeeping genes (Diavatopoulos et al., 2005), and *Pvu*II ribotyping (Register et al., 1997; Register and Magyar, 1999) have proven useful to distinguish among strains of *B. bronchiseptica*. Both methods are highly discriminatory and have been used to infer relationships among isolates and to identify likely sources of exposure. It is unclear which of these methods is most discriminatory since they have not been directly compared using a single set of isolates and relatively few isolates have been typed by more than one method. Comparison among isolates of the GGXXP and PQP repeat regions of the gene encoding the surface-exposed adhesin pertactin (*prn*) can also be an informative adjunct approach since polymorphisms occur at a relatively high frequency (Boursaux-Eude and Guiso, 2000; Register, 2001; Register, 2004). The purpose of this study is to directly compare the discriminatory power of MLST and *Pvu*II ribotyping and to ascertain whether *prn* repeat region sequencing provides an additional level of discrimination using a single set of geographically and genetically diverse strains.

2. Materials and methods

2.1. Bacterial isolates, growth conditions and DNA preparation

A total of 122 isolates of *B. bronchiseptica* was analyzed, selected to represent diverse host and geographic origins. Included among these isolates are representatives of the 22 *Pvu*II ribotypes so far recognized (Register et al., 1997; Register and Magyar, 1999; Register et al., 2000;

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Register et al., 2015a) and 25 of the 61 MLST sequence types (STs) currently associated with *B. bronchiseptica* isolates in the *Bordetella* PubMLST database (http://pubmlst.org/bordetella). Isolates were sourced from the United States, Europe, Israel and Australia and include 24 from humans, 20 from seals, 17 from pigs, 13 from turkeys, 12 from rabbits, 10 from dogs, 8 from guinea pigs, 7 from horses, 6 from cats, 3 from koalas, 1 from a leopard and 1 of unknown origin. All additional information related to the provenance of the isolates can be found in the *Bordetella* Isolates MLST database (http://pubmlst.org/perl/bigsdb/bigsdb.pl?db=pubmlst_bordetella_mlst_isolates; Jolley and Maiden, 2010). Corresponding database isolate ID numbers are 6, 7, 18–24, 27, 69, 71, 72, 75–78, 80, 82–85, 87, 92, 93, 101, 104, 105, 108–110, 113, 118–120, 122–128, 145, 326, 358–370, 379–382 and 444–504.

Bacteria were cultured at 37 °C for 18 to 36 h on Bordet-Gengou agar supplemented with 10% sterile, defibrinated sheep's blood. Chromosomal DNA was purified using a commercially available kit (Promega, Madison, WI) and quantified with PicoGreen reagent (Invitrogen, Carlsbad, CA).

2.2. Ribotyping

*Pvu*II ribotyping was carried out as described (Register and Magyar, 1999). Photographs of *Pvu*II ribotyping gels were scanned and the fragment pattern of each isolate was compared using GelCompar II software (Applied Maths). Similarity between all possible pairs of fingerprint profiles using the coefficient of Dice (Sneath and Sokal, 1973) was calculated by the cluster analysis module of the software. Dendrograms were derived from a matrix of similarity values by UPGMA (unweighted pair group method with arithmetic mean). Ribotype (RT) 1-RT22 have been previously described (Register et al., 1997; Register and Magyar, 1999; Register et al., 2000; Register et al., 2015a). Novel patterns were assigned to newly defined RTs. RTs for 78 of the isolates included in this study have been reported previously (Register et al., 1997; Register and Magyar, 1999; Register et al., 2000; Register, 2004; Rath et al., 2008; Register et al., 2015a).

2.3. Sequence-based typing

PCR amplicons used for sequencing were treated with ExoSAP-IT (USB Corporation) and sequenced directly by fluorescence-based cycle sequencing using Applied Biosystems Big Dye Terminator version 3.1 on an Applied Biosystems 3130 XL Genetic Analyzer at the National Animal Disease Center Genomics Unit. Vector NTI Advance was used for sequence editing and analysis. Final consensus sequences represent a minimum of three-fold coverage with at least one read from each strand.

The scheme of Diavatopoulos et al. (2005) was employed for MLST analysis. MLST allele sequences and profiles were evaluated using the PubMLST *Bordetella* Sequence and Profile Definitions Database, available via a link on the home page of the *Bordetella* PubMLST web site (http://pubmlst.org/bordetella/). For each isolate, amplicon sequences from the seven target genes were concatenated and used to construct a UPGMA tree utilizing the Maximum Composite Likelihood distance estimation model with 1000 bootstrap replicates. All positions containing gaps were eliminated from the dataset (complete deletion option). The final dataset includes a total of 2924 nucleotide positions. Phylogenetic analyses were conducted using MEGA6 version 6.05 (Tamura et al., 2013). STs for 61 isolates included in this study have been reported previously (Diavatopoulos et al., 2005; Rath et al., 2008; Register et al., 2015a). Those for an additional 21 isolates were deduced from genome sequence data (Register et al., 2015b; GenBank accession #NZ_JGWN01000000).

Pertactin gene repeat regions were amplified by PCR, sequenced and analyzed as described (Register, 2004), using the nomenclature of Register (2001) to classify allelic variants. Briefly, the epithet 1-Nx, where N is an Arabic numeral indicating the number of GGXXP repeats and x is a lowercase letter used to distinguish additional unique insertions, substitutions, or deletions, is used to describe region 1 variants. Similarly, region 2 variants are categorized using the epithet 2-Nx, with N representing the number of PQP repeats. An additional Arabic numeral is used to discriminate between different DNA sequence alleles with conservative substitutions that result in identical predicted amino acid sequences. Pertactin repeat region sequences from 86 isolates included in this study were previously reported (Register, 2001; Parkhill et al., 2003; Register, 2004; Diavatopoulos et al., 2005; Rath et al., 2008; Register et al., 2015a) or are available from genome sequence data (Register et al., 2015b; GenBank accession #NZ_JGWN01000000).

2.4. Comparison of ribotyping and MLST

The method of Hunter and Gaston (1988) was used to calculate Simpson's Index of Diversity (SID) for comparisons of the discriminatory power of *Pvu*II ribotyping and MLST.

2.5. DNA sequences

The GenBank accession numbers for sequences newly reported here are KX547658-KX548009.

3. Results

3.1. Ribotype analysis

A total of 32 *Pvu*II ribotype patterns was identified from the 122 *B. bronchiseptica* isolates evaluated based on various combinations of 35 restriction fragments ranging in size from ~1.8 Kb to 5.9 Kb (depicted graphically in Fig. 1). DNA from one isolate, cultured from a guinea pig in Australia, could not be digested completely with *Pvu*II such that an RT could not be assigned. The ten RTs newly identified in this study, RT23-RT32, include both novel restriction fragments as well as novel combinations of previously known fragments.

Cluster analysis of the RT patterns delineates six major clusters (Fig. 2). Clusters I-V were defined previously on the basis of RT1-RT22 (Register et al., 2015a). In the current study, a novel, highly divergent cluster (VI) was identified consisting of RT29 and RT30, each represented by a single human isolate. Cluster VI has <42% similarity to other clusters, which otherwise share a minimum of ~62% similarity. The distribution of the original 22 RTs among clusters I-V was unaffected by the addition of the new RTs reported here but, with the exception of Cluster II, each cluster gained one or more of the newly defined RTs.

The distribution of isolates among RTs and clusters on the basis of the host of origin is detailed in Table 1. Clusters I-V each contain isolates from at least four different hosts while Cluster VI is so far comprised of only human isolates. Because of the limited number of Cluster VI isolates currently available it is unclear whether the cluster is truly host-restricted. Although no other cluster is restricted to a single host, all or a majority of isolates from several hosts are restricted to particular clusters and RTs. Specifically, those from pigs (16/17), turkeys (9/13), dogs (8/10) and cats (6/6) have RTs largely found in Cluster I while all isolates from seals ($n = 20$) are either RT19 or RT22, both within Cluster II. All koala isolates analyzed ($n = 3$) are RT6, in Cluster III, but additional, uniquely sourced isolates are needed to more thoroughly evaluate the strength of this association.

There is no obvious correlation between cluster and geographic origin. Isolates from the United States ($n = 64$) are found in all clusters and those from continental Europe are scattered among clusters I, II, IV and V. Isolates from the United Kingdom ($n = 21$) are found only in clusters I and II but this is largely due to the limited heterogeneity among isolates from seals, the host of origin for 15 of the isolates in the group, all RT19. The remaining RT19 isolate was recovered from a seal off the Danish coast. Of the six Australian isolates evaluated, one is non typeable and the remainder segregate between two relatively divergent