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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*.

Key Words: *Bordetella bronchiseptica*; MLST; ribotyping; pertactin; pubMLST

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; 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/bigddb/bigddb.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 *PvuII* 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 nontypeable and the remainder segregate between two relatively divergent branches of the RT-based phylogeny, with two pig isolates in Cluster I (RT2 and RT3) and three koala isolates in Cluster III (RT6).

3.2 MLST analysis

Thirty STs were identified from the 122 *B. bronchiseptica* isolates evaluated, comprising seven major clades (Fig. 3). Table 2 details the distribution of isolates among clades and STs according to the host of origin. Three or more host species are represented within all clades having at least six isolates. However, isolates from two hosts are restricted to single clades; turkey isolates are found solely in Clade I, with 8/13 typed as ST28, while isolates from seals are either ST33 or ST11, both in Clade IV. Also found predominantly in Clade IV are isolates from pigs (15/17), rabbits (9/12), horses (6/7) and cats (4/6). Isolates cultured from other hosts are more broadly scattered among clades. An exclusive association between ST and host was noted only for ST11, which is comprised wholly of isolates from seals. There is additionally a strong correlation between ST28 and host of origin; with the exception of a single human isolate, all ST28 strains originate from turkeys.

No correlation between clade and geographic origin is evident. Isolates from the United States and continental Europe are found in all clades with six or more members. The two isolates in Clade III were both acquired from koalas in Australia. All but a single isolate from the United Kingdom are in clade IV but, as noted above, this is likely due to the predominance of

seal isolates within that group rather than a geographic association per se. Isolates acquired from Australia segregate between two closely related lineages, Clade III (n=2) and Clade IV (n=4).

3.3 Comparison between ribotyping and MLST

The SIDs for ribotyping and MLST are nearly identical, 0.920 and 0.919, respectively. These values indicate that two randomly isolated strains would be classified as distinct RTs or MLSTs ~92% of the time. Both methods are sufficiently discriminatory for typing purposes based on the recommended threshold of ≥ 0.900 (Hunter and Gaston 1988). However, for ten RTs and eight STs, the alternative method discriminates among isolates that otherwise type identically (Table 3). For six RTs (RT4, RT5, RT6, RT7, RT9 and RT10) and five STs (ST6, ST7, ST10, ST12, and ST33) the alternative method assigns the affected isolates to multiple clades or clusters, often comprising relatively distant branches of the corresponding phylogeny (Figs. 2 and 3; Table 3). For example, RT4 isolates are parsed among MLST clades IV, V and VI, RT5 and RT9 isolates are found in clades IV and VI, RT7 isolates segregate to clades IV and V while RT10 isolates have MLSTs representing clades I and VI. Conversely, ST7 isolates are split among RT clusters I, III, and V, ST10 isolates fall into clusters I, II and V, ST12 isolates are found in clusters I and V while ST33 isolates are represented in clusters II, III and V.

3.4 Pertactin repeat region analysis

The DNA sequences encoding the GGXXP and PQP repeat regions from the *prn* gene were previously made available or were acquired from all but one of the 122 isolates evaluated by MLST and ribotyping. Each isolate was assigned a repeat region allele based on the combined sequence of both regions. Thirty-four alleles were identified (Table 4). Although previously reported repeat region sequences derived from PCR amplicons for isolate 00-P-2730 comprise allele 1-4b1 / 2-6e1 (Diavatopoulos *et al.* 2005), allele 1-4b1 / 2-6a2 is found in

genome sequence data for this isolate (Register *et al.* 2015b). Because other discrepant results have been reported by Diavatopoulos *et al.* (2005), as noted by Register *et al.* (2015a), here we opted to assign allele 1-4b1 / 2-6a2 to 00-P-2730. We did not further investigate the discrepancy, which could arise from either errors in data or reporting or from true sequence differences in cultures of 00-P-2730 maintained by different laboratories.

Only one allele, 1-4a2 / 2-6a1, appears to have a well-supported association with a particular host. Of 17 isolates with that allele all but one originated in seals on the Scottish, English or Danish coasts of the Atlantic Ocean and North Sea. These 16 isolates, which also share the same ST (ST11) and RT (RT19), were collected during a nearly 20-year span of time, from 1988 through 2006. The remaining isolate with a *prn* repeat region allele of 1-4a2 / 2-6a1 was cultured in 1996 from the pneumonic lung of a pig in South Dakota and otherwise types as ST6/RT2. Other ST6 or RT2 isolates from pigs were found among the group studied here (Tables 1 and 2), but none are both ST6 and RT2 (Table 3). The *prn* repeat region allele 1-4a3 / 2-5b1 was found in only two isolates, both from koalas. BLAST queries of *Bordetella* genome and nucleotide sequences currently available in GenBank did not identify any additional isolates having the same *prn* repeat region allele. A third koala isolate has allele 1-3a2 / 2-7a1, found more commonly and in a wide variety of mammalian hosts.

The only isolate for which *prn* repeat region sequences could not be acquired is 99-R-0433, an RT30/ST60 isolate from a human in the United States. Repeated attempts to obtain PCR amplicons encompassing the repeat regions, using a variety of primers, were unsuccessful. Subsequent Southern blotting and restriction mapping suggested only a portion of the 3' end of the gene was likely to be present, with the GGXXP region and most or all of the PQP repeat region missing (K.B. Register, unpublished data). The genome sequence of this isolate was recently obtained (GenBank accession #NZ_JGWN000000000) and provides further insight.

The *prn* open reading frame is predicted to encode an 894 amino acid protein (versus 916 amino acids for the reference isolate RB50), but only ~950 bp at the 3' terminus is recognizable as the pertactin gene. The 5' 1730 bp has only 33.1% nucleotide identity and 9.2% predicted amino acid identity with *prn* from RB50. In contrast, the 3'-terminal 950 bp has 92.9% nucleotide identity and 92.1% predicted amino acid identity with the collinear portion from RB50. Colinearity begins at the extreme 3' end of the PQP region and includes only a single predicted PQP repeat. Accordingly, it is not possible to classify isolate 99-R-0433 on the basis of *prn* repeat regions.

Pairing *prn* repeat region typing with MLST resulted in a total of 47 genotypes and an SID of 0.944, as compared to 0.919 for MLST alone. Of the 13 STs with multiple members, 11 can be further delineated when *prn* repeat region alleles are considered (Table 4). Isolates with ST11 (16 seal isolates) and ST63 (2 koala isolates) could not be further differentiated.

PvuII ribotyping combined with *prn* repeat region typing yielded 54 genotypes and increased the SID from 0.920 to 0.954. This approach resulted in further discrimination of isolates for all except 4 of the 16 RTs represented by more than a single isolate (Table 5). RTs that could not be subtyped when considering *prn* repeat region sequences include RT1 (2 isolates from pigs and 1 from a guinea pig), RT12 (2 rabbit isolates), RT19 (16 North Sea seal isolates) and RT22 (4 Caspian Sea seal isolates).

4. Discussion

Our analyses indicate that MLST and *PvuII* ribotyping are equally discriminatory methods for molecular typing of *B. bronchiseptica*. Because MLST offers many technical and practical advantages over ribotyping, we conclude that MLST is preferable as a primary typing tool. MLST data are objective, interpretation is straightforward and unambiguous and repeat analysis of isolates is rarely needed. In contrast, accurate assignment of ribotypes frequently

requires repeated analysis due to slight run-to-run variability in restriction fragment patterns and the necessity of including appropriate reference standards on every gel. An additional limitation is that some isolates cannot be typed, as seen here. In contrast, MLST is highly standardized, scalable and portable and does not depend on comparisons with previously typed reference isolates. Comparisons of data acquired by different investigators and at different times are easily made, both within and between laboratories. A further benefit is the availability of a publically accessible, curated database serving as a central archive for typing data as well as for isolate-specific provenance and epidemiological information.

Although MLST alone is highly discriminatory, our results demonstrate that isolates sharing the same ST can frequently be further differentiated on the basis of *prn* repeat region sequences. This combined approach was informative for all STs represented by more than a single isolate except for ST11 (n=16) and ST63 (n=2). The ST63 isolates evaluated here were both cultured from koalas in Australia. As there is no additional information available about those isolates, we cannot determine whether there was a physical or epidemiological association between the animals of origin. Given the limited number available for our study, evaluation of additional, uniquely sourced ST63 isolates would better reveal the usefulness of *prn* repeat region sequencing for further distinguishing that ST. Considering the number and origin of ST11 isolates included in our study, it seems doubtful that the inability to subtype the group on the basis of *prn* repeat region sequences is due to insufficient sampling. These 16 isolates, which also share the same RT (RT19), were collected from seals during three successive outbreaks spanning nearly 20 years, from 1988 through 2006, and their geographic origin includes more than 15,000 miles of Atlantic Ocean and North Sea coastlines. Rather, the single *prn* repeat region allele they share is consistent with prior observations documenting a remarkable absence of genetic diversity among *B. bronchiseptica* isolates from seals as compared to isolates from other hosts (Register *et al.* 2015a). ST11/RT19 is the sole *B. bronchiseptica* genotype for which

there is ample evidence of host restriction, having so far been found exclusively in isolates from either common seals (*Phoca vitulina*) or gray seals (*Halichoerus grypus*). The only additional ST represented here by more than a single isolate for which all isolates were acquired from the same host is ST63; both ST63 isolates are also RT6. However, the limited sample size for this ST prevents a sound determination as to whether it may be restricted to a single host.

Identification of an ST7 isolate from a third koala (Table 2) demonstrates that multiple genotypes of *B. bronchiseptica* are capable of infecting koalas.

Our discovery that *B. bronchiseptica* isolate 99-R-0433 has an aberrant *prn* gene lacking the repeat regions was unexpected. Approximately 10 years ago, investigators working with the closely related causative agent of whooping cough in humans, *Bordetella pertussis*, noted the emergence of strains that fail to express pertactin (Bouchez *et al.* 2009). Since that time, pertactin-deficient *B. pertussis* isolates have rapidly expanded worldwide, essentially replacing pertactin-producing strains in some countries, and the defects preventing expression of the gene have been characterized (Bouchez *et al.* 2009; Bowden *et al.* 2014; Pawloski *et al.* 2014; Williams *et al.* 2016). These include single nucleotide substitutions or insertions resulting in frameshift mutations and premature stop codons, the presence of an IS481 insertion sequence between the two repeat regions, promoter inversion and deletions of the promoter and/or a portion of the 5' end of the gene. In every case, both repeat regions are recognizable and intact. A few pertactin-deficient strains of *B. bronchiseptica* have also been identified, some dating as far back as the late 1980's (Register 2004; Diavatopoulos *et al.* 2005). All have one or two nucleotide insertions immediately prior to the GGXXP region, or just within it, that ultimately result in premature stop codons. Among the 122 isolates of *B. bronchiseptica* included in our study are seven that are predicted to be pertactin-deficient (pubMLST database ID numbers 77, 145, 453, 460, 466, 479 and 484); *prn* sequences for two of the seven have been reported previously (Register 2004; Diavatopoulos *et al.* 2005). Six of these isolates possess one of the

same mutations already found in *B. bronchiseptica* while the seventh isolate, 99-R-0433, appears to be missing the majority of the gene, as noted above. Pertactin-deficient strains of *B. bronchiseptica* appear to be rare. Collectively, we are aware of only 13 such isolates, 12 of which have intact repeat regions suitable for typing. The more extensive mutations seen recently in *B. pertussis* have not so far appeared in *B. bronchiseptica* and, with the exception of isolate 99-R-0433, all isolates for which *prn* sequences are available can be classified based on the two repeat regions as described here. Thus, it is expected that utilization of these repeat regions as a basis for subtyping will continue to be a reliable and informative adjunct approach, despite the remarkable degree of genetic variation associated with *prn*. The absence of one or both repeat regions could itself serve as a potentially discriminating feature, should such isolates continue to be only rarely found.

Considering the host of origin for isolates studied here, those from humans appear to possess the most genetic diversity, with representatives in 5/6 RT clusters and 5/7 ST clades, including those most divergent. Isolates from no other host are represented this extensively in either phylogeny. Although a larger number of isolates was evaluated from humans than from any other host, additional studies offer further evidence that the population of *B. bronchiseptica* infecting humans is highly diverse. Ribotyping data collected from prior publications (Register *et al.* 1997; Register and Magyar 1999; Register *et al.* 2000; Register 2004; Register *et al.* 2012; Register *et al.* 2015a) combined with data from this study reveal that 54.2% of human isolates (n=25) comprise unique RTs, as compared to 21.4% from dogs (n=28), 14.1% from rabbits (n=64), 5.1% from seals (n=59) and 3.5% from pigs (n=172). When MLST data from this study are considered together with results from previous reports (Buboltz 2008; Spilker *et al.* 2008) and unpublished data extracted from the *Bordetella* pubMLST database, 47.6% of human isolates (n=85) possess unique STs, versus 45.4% from dogs (n=22), 35.0% from rabbits (n=28) and 26.1% from pigs (n=23). At least some human infections are reasonably ascribed to

nosocomial spread (Stevens-Krebbers *et al.* 1999; Huebner *et al.* 2006; Bose *et al.* 2008), but many others may arise from exposure to non-human sources since humans are not natural hosts for *B. bronchiseptica* (Goodnow 1980). Consequently, the diversity seen among human isolates may be a reflection of the wide host range the bacterium naturally infects. In some cases, typing a human isolate as described here may provide clues for pinpointing sources from which the bacterium was acquired. As an example, the human isolate St. Louis (pubMLST database ID 501) is an ST28/RT15 strain with a *prn* repeat region allele of 1-8a1 / 2-7d1. Five additional isolates in this study share that genotype, all from turkeys in California or in Midwestern states (pubMLST database ID numbers 489-492, 497). Isolate St. Louis was recovered from a 47-year old male with leukemia, residing in Missouri, whose condition rapidly deteriorated due to a respiratory infection. *B. bronchiseptica* was determined to be the causative agent and was recovered from sputum, bronchoscopy specimens and multiple blood cultures (J.P. Lang, personal communication). No additional details are available about this patient but an avian source of exposure seems plausible. A number of reports provide evidence to suggest that many human infections are the result of contact with infected or vaccinated pets (Gueirard *et al.* 1995; Dworkin *et al.* 1999; Foley *et al.* 2002; Berkelman 2003; Rath *et al.* 2008; Register *et al.* 2012; Brady *et al.* 2014; Garcia-de-la-Fuente *et al.* 2015). An example of typing data in support of this concept, acquired here and from a prior study (Register *et al.* 2012), is the finding that of 14 *B. bronchiseptica* isolates known to be ST27/RT4, six were cultured from humans, seven from dogs or cats and one from a pig. Thirteen of the isolates share the *prn* repeat region allele 1-3a1 / 2-8a1; the remaining isolate has allele 1-3a1 / 2-9a1.

In conclusion, our data reveal MLST and *Pvu*II ribotyping to be equally discriminatory methods for genotyping *B. bronchiseptica* and that subtyping on the basis of *prn* repeat region sequences permits higher order resolution of strains. While the application of all three approaches provides the maximum discriminatory power, MLST is the preferred primary typing

method due to its objectivity, scalability, portability, and repeatability. MLST paired with *prn* repeat region typing was demonstrated here to be a highly discriminatory tool suitable for evaluating the population structure and investigating the epidemiology of *B. bronchiseptica*.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Figure legends

Fig. 1. Schematic representation of the *Pvu*II ribotype patterns found among the 122 isolates evaluated in this study.

Fig. 2. Dendrogram and cluster analysis of *Pvu*II *B. bronchiseptica* RTs performed according to UPGMA (tolerance, 1.0%).

Fig. 3. UPGMA tree derived from MLST sequences of 122 *B. bronchiseptica*, isolates evaluated in this study. Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates were collapsed. The percentages of replicate trees in which the associated taxa cluster together in the bootstrap test (1000 replicates) are indicated at each node.

Table 1. Host distribution of *B. bronchiseptica* isolates among ribotypes (RT) and phylogenetically related RT clusters

Cluster	RT	Host												Total
		Human	Seal	Pig	Turkey	Rabbit	Dog	Guinea					Unknown	
								Pig	Horse	Cat	Koala	Leopard		
I	2	0	0	4	0	0	0	0	0	1	0	0	0	5
I	3	2	0	10	0	0	0	2	0	1	0	0	1	16
I	4	7	0	1	0	1	8	0	2	3	0	0	0	22
I	7	1	0	0	0	0	0	0	1	0	0	0	0	2
I	10	1	0	0	0	0	0	0	0	1	0	0	0	2
I	15	1	0	0	7	0	0	0	0	0	0	0	0	8
I	17	0	0	0	0	1	0	0	0	0	0	0	0	1
I	21	0	0	0	1	0	0	0	0	0	0	0	0	1
I	23	0	0	1	0	0	0	0	0	0	0	0	0	1
I	26	0	0	0	1	0	0	0	0	0	0	0	0	1
II	1	0	0	0	0	1	0	2	0	0	0	0	0	3
II	5	0	0	0	0	1	1	0	0	0	0	0	0	2
II	8	0	0	0	0	0	0	0	2	0	0	0	0	2
II	12	0	0	0	0	2	0	0	0	0	0	0	0	2
II	19	0	16	0	0	0	0	0	0	0	0	0	0	16
II	22	0	4	0	0	0	0	0	0	0	0	0	0	4
III	6	0	0	0	0	0	0	0	0	0	3	1	0	4
III	11	0	0	0	0	0	0	1	0	0	0	0	0	1
III	13	0	0	0	0	0	1	0	0	0	0	0	0	1
III	16	0	0	0	1	0	0	0	0	0	0	0	0	1
III	27	1	0	0	0	0	0	0	0	0	0	0	0	1
III	28	2	0	0	0	0	0	1	0	0	0	0	0	3
III	31	1	0	0	0	0	0	0	0	0	0	0	0	1
IV	14	1	0	0	0	0	0	0	0	0	0	0	0	1
IV	18	2	0	1	1	0	0	1	0	0	0	0	0	5
IV	20	0	0	0	1	0	0	0	0	0	0	0	0	1
IV	24	0	0	0	0	0	0	0	1	0	0	0	0	1
V	9	3	0	0	0	5	0	0	1	0	0	0	0	9
V	25	0	0	0	0	1	0	0	0	0	0	0	0	1
V	32	0	0	0	1	0	0	0	0	0	0	0	0	1
VI	29	1	0	0	0	0	0	0	0	0	0	0	0	1
VI	30	1	0	0	0	0	0	0	0	0	0	0	0	1
Total		24	20	17	13	12	10	7	7	6	3	1	1	12

Table 2. Host distribution of *B. bronchiseptica* isolates among MLST sequence types (ST) and phylogenetically related ST clades

Clade	ST	Host												Total
		Human	Seal	Pig	Turkey	Rabbit	Dog	Guinea Pig	Horse	Cat	Koala	Leopard	Unknown	
I	18	1	0	0	0	0	0	0	0	0	0	0	0	1
I	65	0	0	0	1	0	0	0	0	0	0	0	0	1
I	13	0	0	0	0	0	0	0	1	0	0	0	0	1
I	22	0	0	0	0	0	1	0	0	0	0	0	0	1
I	3	1	0	0	0	0	0	0	0	0	0	0	0	1
I	64	0	0	0	1	0	0	0	0	0	0	0	0	1
I	17	1	0	0	0	0	0	0	0	0	0	0	0	1
I	35	0	0	0	1	0	0	0	0	0	0	0	0	1
I	29	0	0	0	1	0	0	0	0	0	0	0	0	1
I	15	1	0	0	0	0	0	0	0	0	0	0	0	1
I	28	1	0	0	8	0	0	0	0	0	0	0	0	9
I	8	2	0	1	1	0	0	1	0	0	0	0	0	5
I	9	1	0	0	0	0	0	0	0	0	0	0	0	1
I	34	1	0	0	0	0	0	1	0	0	0	0	0	2
II	21	1	0	0	0	0	0	0	0	0	0	0	0	1
III	63	0	0	0	0	0	0	0	0	0	2	0	0	2
IV	62	0	0	0	0	0	0	0	0	1	0	0	0	1
IV	23	1	0	0	0	1	0	0	3	2	0	0	0	7
IV	33	0	4	0	0	0	1	0	1	0	0	1	0	7
IV	7	2	0	13	0	2	0	3	0	1	1	0	1	23
IV	12	3	0	0	0	3	0	0	0	0	0	0	0	6
IV	11	0	16	0	0	0	0	0	0	0	0	0	0	16
IV	6	0	0	2	0	2	0	0	2	0	0	0	0	6
IV	14	0	0	0	0	1	0	3	0	0	0	0	0	4
V	27	7	0	1	0	0	5	0	0	1	0	0	0	14
V	31	0	0	0	0	0	1	0	0	0	0	0	0	1
VI	10	0	0	0	0	3	1	0	0	0	0	0	0	4
VI	4	0	0	0	0	0	1	0	0	0	0	0	0	1
VI	5	0	0	0	0	0	0	0	0	1	0	0	0	1
VII	60	1	0	0	0	0	0	0	0	0	0	0	0	1
Total		24	20	17	13	12	10	8	7	6	3	1	1	122

Table 3. Distribution of *B. bronchiseptica* isolates among ribotypes (RT) versus MLST sequence types (ST)

ST	RT																																NT ^a																																																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32																																																								
3	1																																																																																							
4	1																																																																																							
5	1																																																																																							
6	1	1																																	2	2																																																				
7	3	15	1																																2	1																				1																																
8	5																																																																																							
9	1																																																																																							
10	1																																1	1																																																			1			
11	16																																																																																							
12	5																																1																																																							
13	1																																																																																							
14	3																																		1																																																					
15	1																																																																																							
17	1																																																																																							
18	1																																																																																							
21	1																																																																																							
22	1																																																																																							
23	6																																1																																																							
27	13																																1																																																							
28	7																																1																				1																																			
29	1																																																																																							
31	1																																																																																							
33	1																																1	1																																																			4			
34	2																																																																																							
35	1																																																																																							
60	1																																																																																							
62	1																																																																																							
63	2																																																																																							
64	1																																																																																							
65	1																																																																																							

^aNot typeable

Table 4. Pertactin gene repeat region alleles and hosts of origin for MLST sequence types (ST) represented among the isolates evaluated

allele	#bp ^a	ST																												Hosts of origin (n)	
		3	4	5	6	7	8	9	10	11	12	13	14	15	17	18	21	22	23	27	28	29	31	33	34	35	62	63	64		65
1-3a2 / 2-5a1	276					2																									pig (2)
1-3a2 / 2-6b1	282												1																		guinea pig
1-3a1 / 2-6a1	285												3																		guinea pig (2), rabbit
1-3a2 / 2-6a1	285																							1							leopard
1-4b1 / 2-6d1	285																												1		turkey
1-4a3 / 2-5b1	288																											2			koala (2)
1-4b1 / 2-6e1	291																			1					1						turkey, guinea pig
1-3a2 / 2-7a1	294				1	17																		1							pig (7), guinea pig (3), rabbit (2), human (2), dog, cat, horse, koala, unknown
1-3a1 / 2-7b1	297																		6												horse (2), cat (2), human, rabbit
1-4b1 / 2-7e1	297															1															human
1-4a1 / 2-6a1	300				2				3		5													4							rabbit (8), seal (4), human (2)
1-4a2 / 2-6a1	300				1					16																					seal (16), pig
1-4b1 / 2-6a2	300																								1						human
1-3a2 / 2-8e1	303					4																									pig (4)
1-4b1 / 2-7c1	303																		1												dog
1-3a1 / 2-8a1	306		1						1												13			1							dog (7), human (7), cat, pig
1-3a2 / 2-8a1	306				1																										horse
1-5e1 / 2-5c1	306														1																human
1-3a1 / 2-8g1	309			1																	1										horse, cat
1-4a2 / 2-7a1	309				1																										pig
1-4b1 / 2-8b1	309							1																							human
1-4b1 / 2-8f1	309	1																													human
1-5d1 / 2-6a1	315									1																					human
1-3a1 / 2-9a1	318																			1											dog
1-5f1 / 2-6f1	318																										1				cat
1-5c1 / 2-7f1	318																1														human
1-5b1 / 2-8d1	318						3								1																pig, turkey, human, guinea pig
1-5a1 / 2-8c1	321																								1						turkey
1-5b1 / 2-8b1	324						2																								human (2)
1-4a4 / 2-9b1	327																							1							horse
1-6a1 / 2-6c1	342																					1									turkey
1-8a1 / 2-6g1	348																				1										turkey
1-8a1 / 2-7d1	357																				7										turkey (6), human
1-8b1 / 2-8c1	366											1																	1		turkey, horse
Total		1	1	1	6	23	5	1	4	16	6	1	4	1	1	1	1	1	7	14	9	1	1	7	2	1	1	2	1	1	

^a Combined length of GGXXP and PQP repeat regions

Table 5. Pertactin gene repeat region alleles and hosts of origin for ribotypes (RT) represented among the isolates evaluated

allele	#bp ^a	RT																																Hosts of origin (n)
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	
1-3a2 / 2-5a1	276			2																													pig (2)	
1-3a2 / 2-6b1	282																																guinea pig ^b	
1-3a1 / 2-6a1	285	3																															guinea pig (2), rabbit	
1-3a2 / 2-6a1	285						1																										leopard	
1-4b1 / 2-6d1	285																													1			turkey	
1-4a3 / 2-5b1	288						2																										koala (2)	
1-4b1 / 2-6e1	291															1												1					turkey, guinea pig	
1-3a2 / 2-7a1	294		2	10		1	1		1	2		1												1									pig (7), guinea pig (3), rabbit (2), human (2), dog, cat, horse, koala, unknown	
1-3a1 / 2-7b1	297				5			1																									horse (2), cat (2), human, rabbit	
1-4b1 / 2-7e1	297														1																		human	
1-4a1 / 2-6a1	300					1				5		2						1				4			1								rabbit (8), seal (4), human (2)	
1-4a2 / 2-6a1	300		1																	16													seal (16), pig	
1-4b1 / 2-6a2	300																											1					human	
1-3a2 / 2-8e1	303		1	3																													pig (4)	
1-4b1 / 2-7c1	303													1																			dog	
1-3a1 / 2-8a1	306				15			1																									dog (7), human (7), cat, pig	
1-3a2 / 2-8a1	306								1																								horse	
1-5e1 / 2-5c1	306																														1		human	
1-3a1 / 2-8g1	309				1						1																						horse, cat	
1-4a2 / 2-7a1	309			1																													pig	
1-4b1 / 2-8b1	309																												1				human	
1-4b1 / 2-8f1	309										1																						human	
1-5d1 / 2-6a1	315								1																								human	
1-3a1 / 2-9a1	318				1																												dog	
1-5f1 / 2-6f1	318		1																														cat	
1-5c1 / 2-7f1	318																													1			human	
1-5b1 / 2-8d1	318																		3									1					pig, turkey, human, guinea pig	
1-5a1 / 2-8c1	321																				1												turkey	
1-5b1 / 2-8b1	324																		2														human (2)	
1-4a4 / 2-9b1	327								1																								horse	
1-6a1 / 2-6c1	342															1																	turkey	
1-8a1 / 2-6g1	348																										1						turkey	
1-8a1 / 2-7d1	357															6					1												turkey (6), human	
1-8b1 / 2-8c1	366															1									1								turkey, horse	
Total		3	5	16	22	2	4	2	2	9	2	1	2	1	1	8	1	1	5	16	1	1	4	1	1	1	1	1	3	1	0 ^c	1	1	

^a Combined length of GGXXP and PQP repeat regions
^bNot typeable
^cNo *prn* repeat region sequence is available for the single RT30 isolate, 99-R-043

Fig. 1

Fig. 2

Fig. 3