

Full Length Research Paper

The identification of *Bordetella bronchiseptica* from canine isolates of sequencing 16S ribosomal DNA

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The amplification and sequencing of 16S rDNA are useful tools to identify important bacteria that cause diseases in the respiratory tracks of a variety of domestic animals. *Bordetella bronchiseptica*, one of such bacterium, has demonstrated some changes in its metabolic core, generating a greater need for identification through amplification and sequencing of 16S rDNA. We applied this methodology to eleven nasal and pharyngeal isolates from dogs to detect *B. bronchiseptica*. Eight strains were completed from which seven showed 98-99% homology; and three strains were only partially sequenced. All eleven strains sequences were deposited in the GenBank (NCBI). What we found was that the sequencing of *B. bronchiseptica* 16S rDNA is an excellent tool that certifies conventional methods of identification and avoids environmental challenges.

Key words: *Bordetella bronchiseptica*, 16S rDNA, canine bacteria.

INTRODUCTION

Bordetella bronchiseptica is a commensal bacterium that has been isolated from the respiratory tracts of various domestic species, such as pigs, dogs, cats, rabbits, sheep, goats, horses, rats, hamsters and guinea pigs (Dawson et al., 2000; Abdoulaye et al., 2006; Rougier, 2006). It is associated with the clinical diseases atrophic Rhinitis in pigs (Pedersen, 1975; Ross et al., 1997) and Canine Infectious Tracheobronchitis or kennel cough

(Keil and Fenwick, 1998; Mochizuki et al., 2008). This last disease is an illness that affects dogs of all age groups whether house pets or in kennels. Crossed transferences among species, such as dogs to cats (Foley et al., 2002), or rabbits to humans (Gueirard et al., 1995), have been observed. Since *B. bronchiseptica* has also been isolated from humans who suffer from compromised immune responses (Woolfrey and Moody, 1991; Ner et al., 2003;

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Table 1. Strains of *B. bronchiseptica* used in this study.

Strain	Source	Homology* (%)	GenBank access no.
151837083	Pet dog	99	JQ953661
151829626	Pet dog	99	JQ953654
Maxim	Pet dog	98	JQ953662
1518596000	Pet dog	98	JQ953656
MP1	Pet dog	99	JQ953657
231003011	Stray dog	99	JQ953652
331003011	Stray dog	98	JQ953655
361003011	Stray dog	98	JQ953663
151703011	Stray dog	98	JQ953653
78B1	Stray dog	85	JX129161
4F1	Stray dog	99	JQ953651
4617	ATCC†	ND‡	ND‡
10580	ATCC	98	JQ953658

*With respect to sequences already deposited in the GenBank. †American Type Culture Collection. ‡Not determined. The nucleotide sequences were aligned with those available in the GenBank (National Center for Biotechnology Information) data base (www.ncbi.nlm.nih.gov) using the Basic BLAST (Basic Local Alignment Search Tool) software. The sequences were deposited in the GenBank, and accession numbers were issued.

Wernli et al., 2011), it is important to analyze strains that are present in dogs to determine whether they are a source of contagion for people.

B. bronchiseptica is commonly found through conventional methods, that is, colony and microscopic morphology and biochemical profiles (Rath et al., 2008). However, culture conditions may affect the phenotypic and biochemical characteristics, leading to false-negative results.

The use of the polymerase chain reaction (PCR) for the identification of *B. bronchiseptica* has become more widely used. Thus, various primers such as those amplifying insertion sequences and gene coding for alcaligenin *alcA*, (Register and De Jong, 2006; Register and Sanden, 2006) and flagellin gene *flaA* (Hozbor et al., 1999) have been tested; however, the lack of specificity limits their use.

The amplification, sequencing and comparison of the gene coding for 16S ribosomal RNA (16S rDNA) have become useful in bacterial taxonomy. Essential for survival, ribosomal genes are highly conserved in bacteria, and help guarantee success as a means of identification. This genetic material is not as sensitive to environmental conditions as morphology and metabolic signatures.

In a previous work, there were partial sequencing (aprox 500 pb) for 16S rDNA *B. bronchiseptica*, canine gene isolates deposited at GenBank by our group and O'Connor SP (GeneBank Access number NR_024949). We reported a series of eight full sequences and three partial sequences (750-800 pb) after applying the 16S rDNA gene sequencing technology on *B. bronchiseptica* isolates from pet and stray dogs. The aim of this work was to provide several 16S rDNA sequences for *B.*

bronchiseptica. After identification through conventional bacteriological procedures, the amplification and sequencing methodology should provide an accurate identification of *B. bronchiseptica*.

METHODOLOGY

The isolates used in this work are listed in Table 1. From the eleven pharyngeal and/or nasal canine isolates, five were collected from pet and six from stray dogs captured by the Centers for Canine Control (CCC) in the Mexico City metropolitan area. The two reference strains from the American Type Culture Collection (ATCC) were 4617 and 10580.

The isolates were subjected to a panel of biochemical tests that included oxidase, catalase and gelatinase activity, as well as some metabolites such as indol (Cowan and Steel, 1974). Urease activity as well as carbohydrate fermentative metabolites were determined by means of a miniaturized BBL Crystal enteric/nonfermentative ID kit.

Isolates were grown on Brain Heart Infusion (BHI) agar for 48 h at 37°C. A bacterial suspension adjusted to 1.5×10^9 CFU/ml of PBS was centrifuged at 1000 g for one minute. The DNA was extracted using a DNAeasy Blood and Tissue kit, according to the manufacturer's instructions.

The 16S rDNA region was PCR amplified with the universal primer pair RD1 (AAGGAGGTGATCCAGCC) and FD1 (AGAGTTTGATCCTGGCTCAG), according to Louws et al. (1999). 100 ng of total DNA from each isolate was used for the reaction. The reaction buffer contained 20 mM Tris HCl (pH 8), 2.5 mM MgCl₂, 2.5 units of Taq DNA polymerase 0.5 μmol of each primer and 0.2 mM dNTPs.

The mixture was placed in a 2720 thermocycler with an initial denaturation step for 3 min at 94°C. Then, the mixture underwent 30 additional cycles, each consisting of a denaturation for 1 min at 94°C; an annealing for 30 s at 55°C; and an extension for 2 min at 72°C. The reaction ended with a one-step extension for 7 min at 72°C, and it was stored at 4°C. PCR products (20 μl each) were

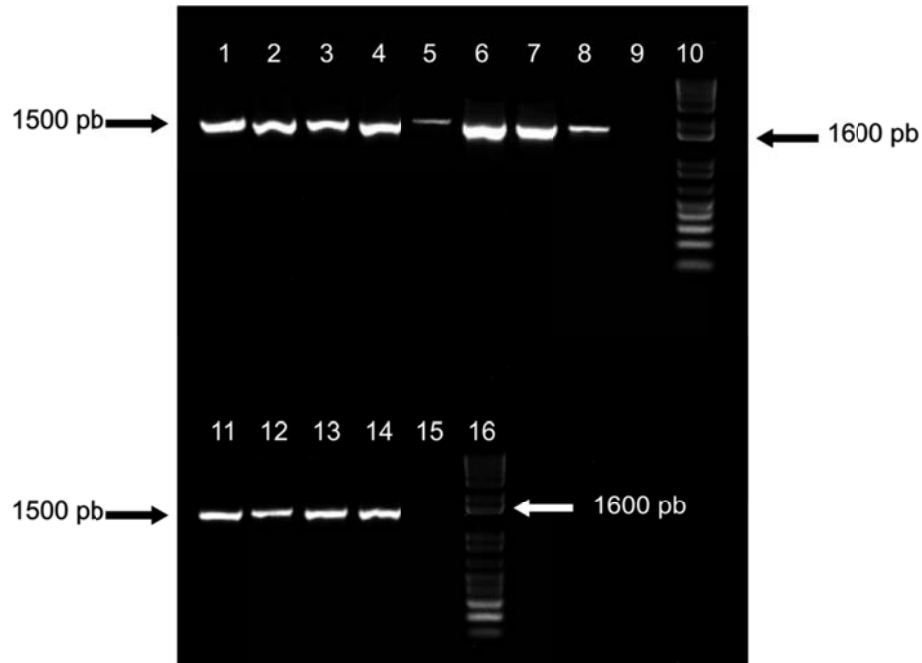


Figure 1. *Bordetella bronchiseptica* strains 16S rDNA PCR products. Lanes: 1– 231003011, 2– 151703011, 3– 331003011, 4– 361003011, 5- 10580 ATCC, 6- 78B1, 7- VIH6, 8- 151837083, 10- Biomarker 1Kb, 11- 151829626, 12- Maxim, 13- 1518596000, 14- MP1, 15- negative and 16- Biomarker 1 Kb. Lane 7 shows the PCR product from a DNA isolate that is not included in this paper.

analyzed by 1% agarose gel electrophoresis, visualized and documented.

For sequencing, the PCR products were purified with Wizard kits, according to the manufacturer's instructions. The amplicons were sequenced using a 3100 Genetic Analyzer. Nucleotide sequences were compared with those available in the GenBank (National Center for Biotechnology Information) database (www.ncbi.nlm.nih.gov) using Basic BLAST (Basic Local Alignment Search Tool) software.

Comparison of 16S rDNA regions V4-V5 (Sun et al., 2013), were performed using the ClustalW software (Larkin et al., 2007).

RESULTS AND DISCUSSION

All isolates, including the ATCC reference strains, were identified morphologically as *B. bronchiseptica*, this is, all showed Gram-negative coccobacillary microscopic morphology. Most of the strains, certainly the ATCC reference strains, complied with the whole metabolic pattern and the corresponding enzyme activities (Cowan and Steel, 1974). They expressed oxidase and catalase activities and degraded citrate. But, they did not ferment any of the ten carbohydrates tested (arabinose, mannose, sucrose, melibiose, rhamnose, sorbitol, mannitol, adonitol, galactose and inositol), neither did they produce indol. The strains showed urease activity, although in some isolates the activity was uncertain even after 48 h of incubation. Since this enzymatic activity is a key property that distinguishes *B. bronchiseptica* from other

species within its genus (Dénes et al., 2006), the expression was corroborated with the Christensen's tube method (MacFaddin, 2000). These measurements indeed confirmed very low quantities of urease. It is important to point out that urease activity may be regulated by culture medium composition, for example, by some $MgSO_4$ salts (Dénes et al., 2006). Urease activity is one aspect of the metabolism of *B. bronchiseptica*, which occasionally generates misleading results when using only a biochemical and metabolic criteria, raising the need of molecular identification or confirmation as described in this paper.

The 16S rDNA is a gene present in all bacteria with 1500 bp coding for a portion of the 30S ribosomal subunit. The analysis of 16S rDNA has been widely used to establish the phylogenetic relationships among bacterial groups (Garrity et al., 2005; Trüper and Schleifer, 2006). The eleven isolates of *B. bronchiseptica* and the two ATCC reference strains produced an amplicon of 1500 bp with the primer pair FD1 and RD1 described by Louws et al. (1999) (Figure 1). From the eleven isolates, eight complete sequences were obtained and submitted to GenBank for identification. The remaining three sequences were obtained with one of the primers (FD1), with average sizes of 750 to 800 bp and were also submitted. The sequences were deposited in the GenBank under the following accession numbers: JQ953661-63, JQ953651-57, JQ9536559 and JX129161 (Table 1). All but one of the whole sequences showed 98-

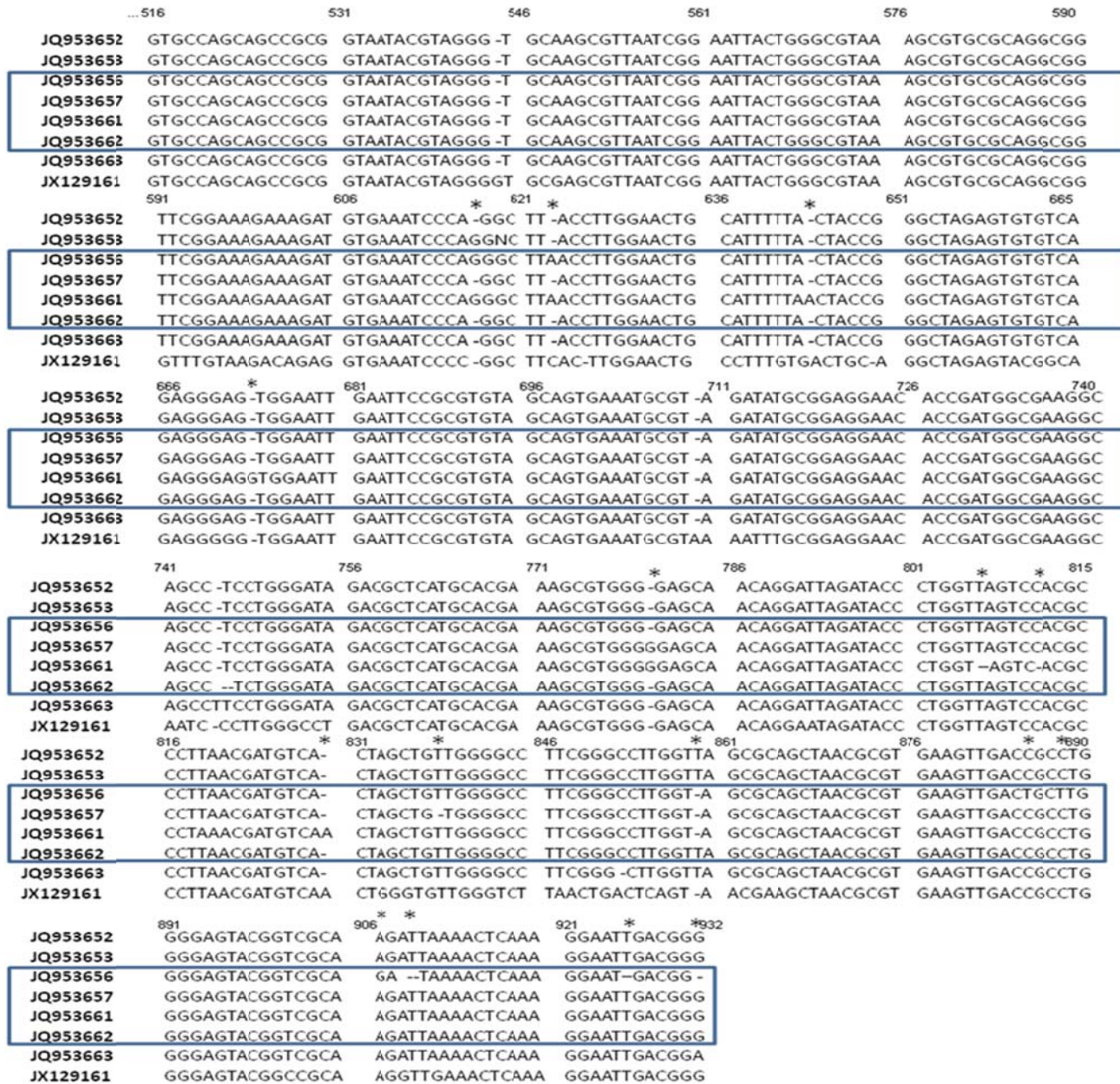


Figure 2. *Bordetella bronchiseptica* V4-V5 16S rDNA alignments. Regions V4-V5 (nucleotide 516 and so on) from 16S rDNA whole sequences from *B. bronchiseptica* were aligned by ClustalW. Sequences belonging to *B. bronchiseptica* 16S rDNA from pet dogs are embedded in square boxes. Differences are marked by *.

99% homology with *B. bronchiseptica*. The strain 78B1 showed 84% homology. The S16 rRNA has been used as a taxonomic tool because it interacts with its linking proteins and keeps the ribosome functioning. Horizontal transfer is considered to be highly unlikely for these genes; however some studies challenged that assumption (Kitahara et al., 2012). A thorough study by Sun et al. (2013) compared the intragenomic variation of the 16S rDNA with a substantial database made of sequences from Archea and bacteria. They found that indeed 16S rDNA is a conserved gene, although its mosaic design

allows for determining whether the higher variability is within the gene sequence. The fragments named V4-V5 (515-533 and 907-927, respectively) are suggested to be appropriate to analyze sequences from suspected strains from the same species (contain the least intragenomic heterogeneity). In Figure 2, we compared the regions V4-V5 from the eight full sequences and observed that seven out of eight contained variations below the higher intragenomic heterogeneity (under 87%). This is in agreement with an adequate variability among strains found by Sun in his 2013 sequences comparison study. The strain 78B1

was slightly under such value (86%).

The comparison of 16S rDNA sequence between pet and stray dogs seems to indicate little differences. When comparing regions V4 and V5 (Sun et al., 2013) from stray and pet dogs, we observed in three out of four cases a deletion at nucleotide 859. In two of the pet-dog sequences, we also observed insertions of adenine at nucleotide 623 and guanine at nucleotides 617 and 780. Several deletions were also found at nucleotide sites: 806, 811, 838, 908, 909, 926 and 932. Insertions of adenine were noted at nucleotide sites: 644, 673 and 830, and three sets of transitions T/C (nucleotide 886 and 888) or G/A (nucleotide 906).

The strain 78B1 was classified as *B. bronchiseptica*, despite having only 84% homology with strains previously registered in GenBank. Homology of V4-V5 region was 86%, which is only 1% below the suggested strain variation. At first the GenBank itself suggested a possible “chimeric” sequence, that is, the product of a contaminated culture. In response, the electropherogram of the strain was revised, and it was verified that there was no contamination. After the sequence was resubmitted, the strain was determined to belong to *B. bronchiseptica*. Exceptions to the established criterion of sequence homology equal to or greater than 97% for bacteria have been reported previously (Janda and Abbott, 2007; Petti, 2007). Such is the case of *Clostridium tetani* and *Clostridium innocuum*, which shows a 20% difference in the 16S rDNA gene sequence (Clarridge, 2004).

The biochemical allocation followed by a molecular analysis of 16S rDNA provides specific evidence that *B. bronchiseptica* belongs to the species. The information gathered from this work will enrich the data belonging to *B. bronchiseptica*. Rarely found in humans, *B. bronchiseptica* was isolated from an immunocompromised person who was suspected of contracting the bacteria through contact with a pet (Wernill et al., 2011). We were not able to compare pet 16S rDNA sequences to any *B. bronchiseptica* isolated from humans. There is only one partial sequence that is not long enough to contain the V4-V5 regions.

In the present study, we established the identity of *B. bronchiseptica* strains from dogs isolates first through canonical microbiological methods, which narrows the identification to species followed by 16S rDNA sequencing to guarantee the strains' identity. Such methods combined in that specific order would diminish confusion with other closely related species with strains. Furthermore these sequences widen data bank information for the *B. bronchiseptica* 16S rDNA. A rich database for 16S rDNA is necessary for gene sequence-based identification schemes, that is, MicroSeq 500 (Woo et al., 2001); which offers the best alternatives in identifying strains lacking typical biochemical profiles.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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