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Identification of *Brucella canis* Group 2 in colombian kennels¤

Identificación de Brucella canis Grupo 2 en criaderos de perros en Colombia

Identificação de Brucella canis Grupo 2 em criadouros de cães na Colômbia

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Summary

Background: recently, isolates of *Brucella canis (B. canis)* were classified into two groups, Group 1 and Group 2, based on a 794 bp deletion in the polysaccharide deacetylase gene in Group 1, or absence of this deletion in Group 2. In Colombia, *B. canis* was first isolated from sick or clinically healthy seropositive dogs in 2005. **Objective:** to determine *Brucella canis* genotype in isolates from Medellín Metropolitan Area. **Methods:** 49 isolates from a study involving 193 dogs in 10 kennels were analyzed by Polymerase Chain Reaction (PCR) using three primer pairs of eight previously reported. **Results:** all isolates were positive for 152, 272, and 794 bp fragments, indicating that they belong to Group 2. **Conclusion:** this article presents strong evidence based on the new molecular marker used to classify *B. canis* isolates from Medellín (Colombia) into Group 2. This is a pioneer molecular study on the presence of *B. canis* in Colombia and it would be interesting to apply this method to characterize the infection epidemiology and explore its domestic clinical dynamics.

Key words: *molecular marker, PCR, zoonoses.*

Resumen

Antecedentes: recientemente, las cepas de *Brucella canis (B. canis)* fueron clasificadas dentro de dos grupos, Grupo 1 y Grupo 2, de acuerdo a la presencia o ausencia de una deleción de 794 bp en el gen de la polisacárido deacetilasa, respectivamente. En Colombia *Brucella canis* se aisló por primera vez en 2005 de perros seropositivos, enfermos o clínicamente sanos. **Objetivo:** determinar el genotipo de aislamientos de *Brucella canis* provenientes del Area Metropolitana de Medellín. **Método:** 49 aislamientos de un estudio con 193 perros en 10 criaderos fueron sometidos a tres Reacciones en Cadena de la Polimerasa (PCR) simple, empleando 3 pares de cebadores de 8 previamente reportados**. Resultados:** todos los aislamientos amplificaron los fragmentos de 152, 272 y 794 bp, indicando que pertenecen al Grupo 2. **Conclusiones:** este

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artículo presenta evidencia sólida para clasificar las cepas de *Brucella canis* aisladas en Medellín (Colombia) en el Grupo 2, basado en el uso del nuevo marcador molecular. Este es un estudio molecular pionero de *B. canis* en Colombia y sería interesante aplicar este método para caracterizar la dinámica epidemiológica y clínica de la infección en el país.

Palabras clave: *marcador molecular, PCR, zoonosis.*

Resumo

Antecedentes: atualmente, as cepas de *Brucella canis* foram classificadas em dois grupos, chamados de grupo 1 e 2, de acordo com a presença de uma deleção de 794 pb em seu genoma. No ano 2005, isolaram-se a *Brucella canis* pela primeira vez na Colômbia, obtida de cães doentes e soropositivos. Neste artigo descrevem-se a classificação de cepas de *B. canis* isolados em Medellín (Colômbia), de acordo com a nova norma. **Objetivo**: determinar o genotipo de isolamentos de *Brucella canis* a partir de amostras da Área Metropolitana de Medellín. **Métodos**: neste estudo utilizaram-se 49 isolamentos obtidos de 193 cães de 10 criadouros diferentes, os quais foram submetidos para três analises de Reação em Cadeia da Polimerase PCR simples com a utilização de três pares primers dos anteriormente informados. **Resultados**: todos os isolamentos analisados amplificaram fragmentos de 152, 272 e 749 pb, comprovando que pertencem ao grupo 2. **Conclusões**: este artigo apresenta evidencia solida para clasificar as cepas de *Brucella canis* isoladas em Medellín (Colombia) no grupo 2, baseados no uso do novo marcador molecular. Seria de grande importância que os estudos de epidemiologia molecular ampliarem-se para outras áreas de pesquisa na Colômbia, com a finalidade de seguir os patrões das infecções e fazer as correlações clinicas.

Palavras chave: *marcador molecular, PCR, zoonose.*

Introduction

Brucella canis (*B. canis*) bacteria target androgen-dependent organs, causing reproductive failure and infertility in dogs. It was first isolated in Colombia from seropositive sick dogs in 2005 (Giraldo *et al*., 2009). This pathogen was also isolated from an owner whose kennel tested positive (Olivera and Di Lorenzo, 2009). So far, 49 isolates from 10 kennels and one isolate from a person in Medellin have been identified in Colombia. A deletion in a specific region of certain *B. canis* genomes can occur, consequently affecting their band pattern and differentiating them from *Brucella suis* (*B. suis*) (García-Yoldi *et al*., 2006). Such isolates have been classified as *B. canis* Group 1. Isolates not presenting this deletion are classified as *B. canis* Group 2 (Koylass *et al*., 2010). The objective of this communication is to report that *B. canis* isolates from Medellin metropolitan area belong to the Group 2 genotype. Our results suggest that this is currently either the predominant or the sole group in this region of Colombia.

Materials and methods

Bacterial isolates, strains, and DNA extraction

A set of 49 *B. canis* isolates from blood were used. Isolates were identified by biochemical tests and confirmed by Argentina's Administración Nacional de Laboratorios e Institutos de Salud *"Dr. Carlos G. Malbrán*" (Malbrán Institute). Dogs located throughout Medellín's metropolitan area were used in this study. Genomic DNA was extracted using the boiling method (Queipo-Ortuño *et al*., 2008) where five colonies were picked from tryptic soy agar (Difco, BD Diagnostic Systems, Sparks, MD, USA), cultured and suspended in 200 µl distilled water aliquots, boiled for 10 min, then centrifuged for 5 min at 20.000 x g. The resulting supernatants were used for PCR. The DNA concentration and quality was measured by the Nanodrop system at 260/280 (Rossetti *et al*., 2009).

Primer selection

Extracted DNA, purified from the isolates, was amplified in three simple PCRs using three primer pairs selected from the eigth used by García-Yoldi *et al*. (2006). Primer sequences are presented in table 1.

Table 1. Primer sequences used.

Primer	Sequence
BMEII0987f	CGCAGACAGTGACCATCAAA
BMEII0987r	GTATTCAGCCCCCGTTACCT
BR00953f	GGAACACTACGCCACCTTGT
BR0953r	GATGGAGCAAACGCTGAAG
BMEI1436f	ACGCAGACGACCTTCGGTAT
BMEI1435r	TTTATCCATCGCCCTGTCAC

The first primer pair used was the genus-specific BMEII0987f and BMEII0987r, which amplified a 152 bp fragment in all *Brucella* species except *B. neotomae.* The second pair consisted of primers BR00953f and BR0953r, which amplified a 272 bp fragment in *B. canis, B. suis,* and *B. neotomae.* The last pair was the species-specific BMEI1436f and BMEI1435r, which amplify a 794 bp fragment in *B. suis,* but not in *B. canis* ATCC 23365.

PCR procedure

The PCRs were performed using 25 µl containing 3 µl of 1 ng/µl DNA template, 0.625 µl of 0.25 µM of each primer, 0.2 µl of *Taq* DNA polymerase (5 UI/µl, Fermentas Taq DNA polymerase recombinant, Foster City, CA, USA), 3 µl of 3 mM $MgCl_2$ (Fermentas), 2.5 µl of 10X buffer with Tris-HCl, Triton X-100, and KCl (pH 8.8) (Fermentas), 0.5 µl of 10 mM of each dNTP, completing with distilled water. Amplification was run in a PTC 200 thermocycler (Perkin-Elmer Inc., San Jose, CA, USA) for 25 cycles, as reported by García-Yoldi *et al*. (2006). Water was added in the negative control, and DNA from the attenuated Carmichael *B. canis M-* (a less mucoid *B. canis* strain used as antigen for serologic diagnosis of canine brucellosis) served as positive control (Carmichael and Joubert, 1987). The PCR products were analyzed in 1% agarose gels containing 0.5 µg/ml ethidium bromide and detected under UV light using a photo-gel imaging system (Transilluminator Mini Benchtop Model *M-10E,* UVP, Upland, CA, USA).

Sequencing of 794 bp amplicon

The 794 bp amplicon of the 12 genes *virB* from 10 field isolates was automatically sequenced using the forward primer by Macrogen Company (Korea). Sequencing was conducted using the BigDye™ terminator kit (ABI, Foster City, CA, USA) cycling conditions (Tiller *et al.,* 2010). The reaction products were purified by ethanol precipitation and analyzed with the automatic sequencer 3730XL (www.macrogen.com). The 794 bp fragment sequences were analyzed using the ClustalW software (Hall, 1999). These sequences were compared with the sequences reported in GenBank for *B. canis* ATCC 23365 and *B. canis* HSK A52141 (Figure 2).

Results

The three fragments (152, 272, and 794 bp) were amplified by PCR in all 49 isolates (Figure 1).

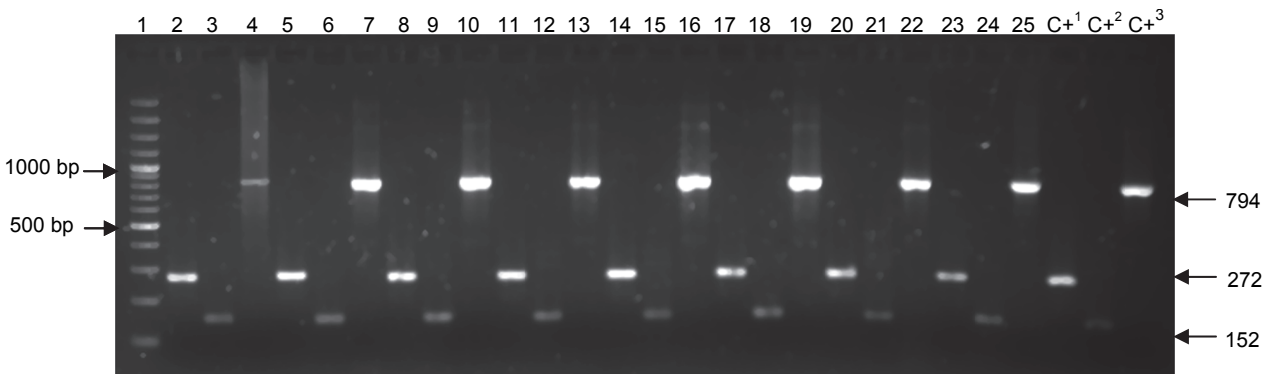

Figure 1. Amplification of the 3 PCR products (152, 272 and 794 bp) of 8 out of 49 different *B. canis* isolates from dogs in Medellin, Colombia. Lane 1: molecular weight markers, Generuler™ 100 bp plus DNA ladder, 100-3000 bp (Fermentas Inc., Burlington, Canada). Lanes 2-4: isolate 1. Lanes 5-7: isolate 2. Lanes 8-10: isolate 3. Lanes 11-13: isolate 4. Lanes 14-16: isolate 5. Lanes 17-19: isolate 6. Lanes 20-22: isolate 7. Lanes 23-25: isolate 8. Lanes $C+^1$, $C+^2$ and $C+^3$ correspond to the positive controls 152, 272 and 794 bp.

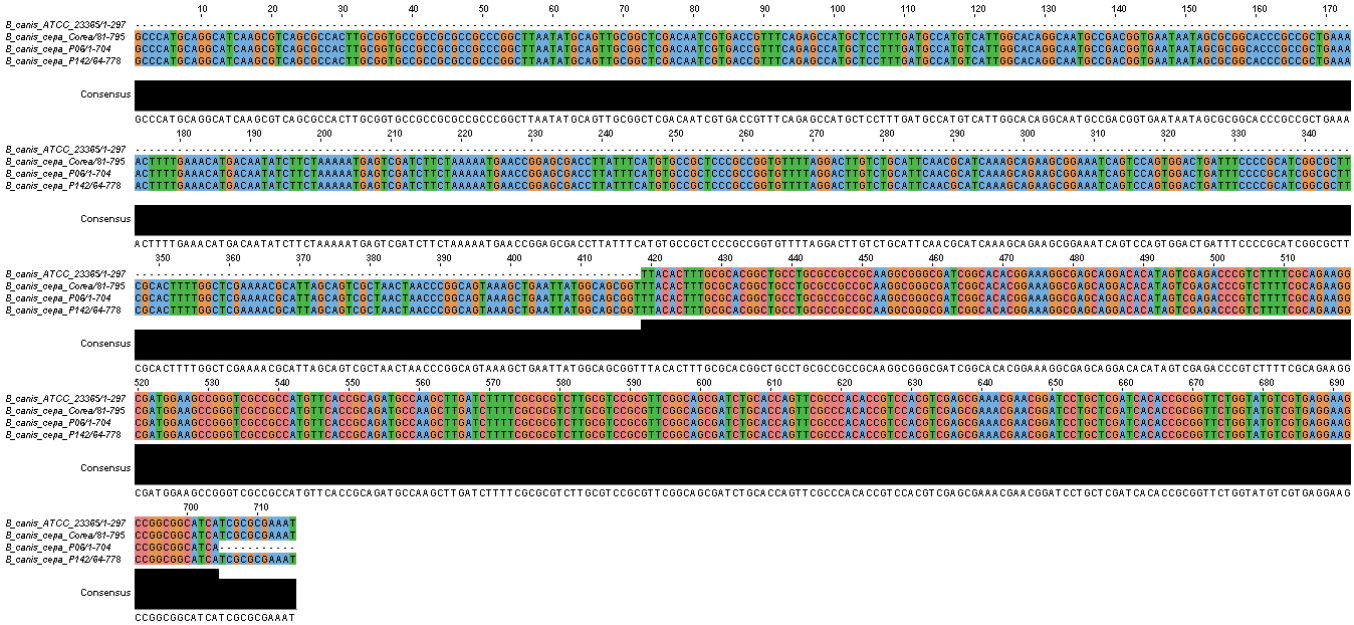

Figure 2. Multiple nucleotide sequence alignments of two *B. canis* isolates from Colombia classified in Group 2, compared to the sequence of *B. canis* ATCC 23365 and *B. canis* HSK A52141 (Corea strain) strains. Line 1 corresponds to the sequence of *Brucella canis* ATCC 23365, line 2 corresponds to the sequence of *Brucella canis* HSK A52141, and lines 3 and 4 correspond to isolates P06 and P146 from Colombia. The consensus region is represented with a black line. *B. canis* ATCC 23365 does not have this region, which is represented as a pointed line between nucleotides 1 to 418. Nucleotides are represented by colors: Cytosine (C) pink, Thymine (T) green, Adenine (A) blue, and Guanine (G) orange.

DNA sequence analysis of the 794 bp fragment

The sequences obtained in this study revealed high homology with *B. canis* HSK A52141, but not with *B. canis* ATCC 23365, which presents a deletion in the region of polysaccharide deacetylase gene and does not align with the other strains, as shown in figure 2.

Discussion

When a new canine pathogen was isolated from dog abortions in 1969, the question arose as to whether this bacterium should be designated as *B. suis* biotype 5 or *B. canis*. Eventually these isolates were designated *B. canis* due to the lack of lipopolysaccharide antigen associated with smooth agglutinogen and endotoxin, and also because it does not utilize erythritol (Jones *et al*., 1968); thus differing significantly from *B. suis*. This classification was adopted by the International Committee on Bacteriological Nomenclature's subcommittee on taxonomy of *Brucella* for the *B. canis* strains RM6/66 ATCC 23365 and NCTC 10854 (Banai and Corbel, 2010).

For many years the only listed strain in GeneBank was *B. canis* ATCC 23365. This made the molecular classification of *B. canis* strains difficult due to existing similarities between *B. canis* and *B. suis* and their high degree of genetic homology. Clarification was offered by García-Yoldi *et al*. (2006) who published a method for identifying and differentiating all *Brucella* species using a deletion in the *B. canis* genome that differentiates it from *B. suis*. However, Koylass *et al.* (2010) demonstrated that not all *B. canis* strains have this deletion. Currently, two *B. canis* groups are recognized: Group 1, which carries the deletion, and Group 2, which lacks it. This gap is due to the impossibility for the primers that amplify the 794 bp fragment to anneal to this region. The 49 isolates tested in the current study do not present the deletion. Therefore, we concluded that the isolates studied in Medellin belong to *B. canis* Group 2 genotype.

Amplification of the 794 bp product suggests that we are in the presence of either *B. suis* or *B. canis* Group 2. The likeliness that *B. suis* was the

infectious agent in our set of isolates was ruled out because these isolates were obtained from dogs and they were biochemically typified as *B. canis* by the internationally recognized Malbrán Institute (Argentina).

Kim *et al*. (2011) sequenced the *B. canis* HSK A52141 strain. It differs from the ATTTCC prototype and, as shown in figure 2, the alignment of the corresponding fragment in our study indicates complete homology, indicating that *B. canis* HSK A52141 and our isolates belong to the same group. On the other hand, Gyuranecz *et al*. (2011) recently reported that some dog isolates, initially confused with *B. suis* when studied by the Bruce-ladder technique, were finally identified as *B. canis* Group 2 when analyzed by single nucleotide polymorphism (SNP).

Clinical, pathological, or epidemiological canine brucellosis patterns have not been characterized in Colombia, notwithstanding the fact that the bacterium's presence has been documented. Standardization of serological, bacteriological, and molecular techniques is very important for these purposes as well as for basic phylogenetic studies and to compare cross antigenicity and pathogenicity of Group 1 and 2 in dogs and human isolates.

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