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RESEARCH ARTICLE

Differential Detection of Brucella Canis by Means a Conventional Polymerase Chain Reaction

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Abstract

The canine Brucellosis (CB) is an infectious, contagious and zoonotic disease caused by Brucella canis (B. canis). Nevertheless, there have been described sporadic infections caused also by *Brucella suis* and *Brucella abortus* as also for Brucella melitensis which produce an auto limited illness. This infection is characterized for producing infertility in males and females, affecting the reproductive life of the animal, which leads to important economic losses in breeding-kennels and affective loss for owners when the dog it carried to euthanasia. The diagnosis is usually realized by means serological tests whose principal disadvantage is the production of false positive results due to crossed reactivity with other bacteria, of the same or different genus, or false negative results in chronic infection cases and for this reason the diagnostic confirmation by means bacterial isolation is necessary. Nevertheless, the previous carries out risk of infection to the laboratory personnel due to zoonotic character of these bacteria. Additionally, this procedure involves a long period of incubation.

In consideration to the previous antecedents, the objective of this work was to develop a conventional PCR capable of differentiating between the detection of Brucella canis, Brucella sues and Brucella abortus, using the *in silico* design of optimal primers, for generating a different and minor size fragment for Brucella sues with sequencing validation. Furthermore, the nucleotide sequences analysis was realized by means of free access programs.

This way, this method will constitute a promising alternative to the bacterial isolation as diagnostic direct and complementary method to serological tests.

Key words: Brucella canis, conventional PCR, in silicon design (primers)

Introduction

Canine brucellosis (CB) is an infectious disease of chronic course and worldwide distribution caused by four species of the Brucella genus: Brucella canis (B. canis), Brucella melitensis (B. melitensis), Brucella Suis (*B. suis*) and *Brucella abortus* (*B. abortus*). Of all, the first is the species of epidemiological importance in the dog, since the three remaining species produce occasional infections in some individual cases [1, 2] and a self-limiting illness [3, 4]. Most of the infections are not diagnosed only by the antecedents or routine physical examination [4] and for this reason, it is necessary to have a clinical, serological and bacteriological background, to reach the definitive diagnosis [1].

The clinical manifestations are very variable, with the reproductive tract being the most affected. In females, late abortion or cases of puppies that die after a few hours and up to a month after birth are described, or survive the infection and develop the disease later. In males there is epididymitis, prostatitis, and in chronic cases, testicular atrophy. Testicular damage initiates an autoimmune response characterized by the production of anti-sperm antibodies that contribute to infertility [1]. Non-specific signs, in both sexes, include lethargy, generalized lymphadenopathy, disco-spondylitis, meningoencephalitis and recurrent anterior uveitis [4, 5].

Sexual transmission is the main route of infection among adult dogs of different sex, constituting an important risk of infection at the time of crossing, while in puppies, it is mainly via or nasal through direct or indirect contact with urine, semen, aborted material, vaginal secretions and milk [6]. In addition, congenital or intrauterine transmission acquires an important role in the dissemination of the infection to puppies [4]. This is even more important when considering that vaccines are not available [5] or completely effective antibiotic treatment [1].

BC causes significant economic losses in dog breeding sites, since infected animals cannot be used for crossbreeding [1]. Thus, euthanasia remains the main control strategy [5] and for this reason it is essential to have a sensitive, specific and rapid diagnosis to obtain reliable results. For the laboratory diagnosis exist several microbiological, serological and

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molecular drawbacks such as extending time, cross-reaction and very similar genome sequences. In this last case, several methods of DNA amplification have been developed, such as the Polymerase Chain Reaction (PCR) that has been shown to be confirmatory in the diagnosis of brucellosis [7, 8, 9]. In addition, PCR has high values of sensitivity and diagnostic specificity. Thus, a sensitivity and specificity of 86.6% and 100%, respectively, have been determined in seminal samples, these values increased even more when the sample corresponded to blood, where both reached 100% [7].

However, the most important challenge in using PCR for the identification of different Brucella species is their high percentage of genomic identity, greater than 94%, with a much narrow nucleotide relationship between Canis and B. suis [10]. Despite this high percentage of genomic identity, [11] developed a multiple PCR, called Bruce-ladder multiplex PCR, capable of identifying and differentiating all species and vaccine strains of the genus Brucella. Thus, B. canis is identified and differentiated from the rest by default (absence of an amplicons of 794 base pairs (bp). Unfortunately, in this PCR protocol some strains of B. canis presented the amplicons of 794 bp, incurring an erroneous differential diagnosis between B. canis and B. Sui, which was later corrected by [12], able to unequivocally distinguish B. canis from B. suis by absence of amplicons 766 bp Although B. suis is not found in our country (OIE, 2013), it is considered convenient and interesting to include the DNA of this Brucella species in the present study, since it represents the greatest challenge when molecularly differentiating it from B. canis its greatest nucleotide relationship [10].

Due to advantages of the PCR technique in terms of sensitivity, specificity and speed in obtaining results, this report considered the in silicon design of a new pair of primers and its application in a conventional PCR protocol that allowed the obtaining an amplicons of compatible size only with the presence of B. canis, establishing a differential diagnosis by effect (unlike the method of [12]. This was validated by the existence of a genomic zone not conserved within the genome of B. canis (see Annex 1) considering the nucleotide sequences of the reference strains published in GenBank®: B. canis ATCC 23365 (NC_010103.1), B. suis 1330 (NC_017251.1), B. abortus S19 (NC_010742.1) and bv.1 str. 9-941 (NC_006932.1) and B. melitensis bv.1 str 16M (NC_003317).

Materials and Methods

The present work was done in the laboratories of Microbiology and Virology from the Department of Animal Preventive Medicine of the Faculty of Veterinary and Animal Sciences of the University of Chile (FAVET).

The samples for the implementation of this PCR technique, DNA samples of different origin and according to availability of bacterial strains were used from the respective laboratory: DNA from a strain of B. canis isolated in our laboratory was used as a positive control from a dog belonging to the group "4A" (Aid to the Abandoned Animal Association) of the FAVET maintained at refrigeration and freezing temperatures. DNA controls of B. abortus, B. Sui, Salmonella Enteritis's and Escherichia coli were used as negative controls. The positive control and the negative controls, with the exception of B. Sui, were transferred by Dra. Consuelo Borie from our laboratory, in culture broth with a concentration greater than or equal to 105 CFU / ml, and killed by thermal treatment. The negative control, DNA from B. Sui, was provided by Dr. Silvio Cravero (Institute of Biotechnology, INTA, Argentina). Nuclease-free water was used as reagent control.

The design of primers for B. canis was done by using the Geneious v4.8 software, which uses official information from GenBank®. The nucleotide sequence of B. canis ATCC 23365, B. Sui ATCC 1330, B. abortus S19 and bv. 1 str. 9-941 and B. melitensis bv.1 str. 16M, were submitted to nucleotide alignment to identify a less conserved area within the genome of B. canis in relation to the other remaining strains (Figure 2), once this was detected, a pair of primers was designed in silico (BC1 and BC2), whose sequences are the following: BC1: 5'-ACGAACACAA GGGCCAATAC-3'and BC2: 5'-GGACGGCTACAAGATCGAAG-3'. Once the primers were designed, they were sent to synthesize the BIOSCAN Company.

The reaction mixture (in triplicate) consisted in 5 µl of bacterial DNA, 15 µl of a 2X PCR Master Mix Ferments® kit (including the thermos table polymerase, the deoxynucleotide triphosphates (dNTP), the buffer of reaction and MgCl2) and 5 µl of each primers, obtaining a total volume of 30 µl. The protocol for the amplification consisted of an initial denaturation at 94ºC for 1 minute and then 33 cycles consisting of a denaturation at 94ºC for 1 minute, alignment at 55ºC for 1 minute (determinate using temperature gradient thermo cycler) and elongation at 72ºC for 1 minute. Finally, an elongation at 72ºC for 5 minutes.

The visualization of the amplified product was carried out by electrophoresis in 3% agarose gel (Winkler ®) in Tris acetate EDTA (TAE) buffer (Fermentas®). The PCR product was mixed with 6 µl of the commercially available 6X Mass Ruler Loading Dye Solution product (Fermentas ®), which contains glycerol to increase the sample density, and bromophenol blue to observe the migration front. Electrophoresis was carried out at 90 V for 40 minutes, using Hyper ladder TM IV (Bio line®, 50-1000pb) as molecular size marker. After, the gel was incubated in ethidium bromide (0.5 µg / mL) (Sigma®) for 40 minutes and photographed on an ultraviolet transluminator (Transluminator UVP ®). The DNA fragments obtained in the PCR were sent in triplicate to the company Genetic for the determination of its nucleotide sequence. The sequences obtained were aligned using the Crustal Ω program initially to obtain a consensus sequence and subsequently was entered into the BLAST program (Basic local Alignment Search Tool) in order to determine the percentage of nucleotide identity (PIN) of the amplified fragment.

Limited access to the facilities was considered, to have an

exclusive area of work for the realization of the PCR, use of Bunsen burner to delimit a bio clean work area, all this in order to avoid a possible contamination with genetic material other than the DNA of interest. In addition, clean apron and disposable latex gloves should be worn. For the visualization of the product it was necessary to use disposable latex gloves, since ethidium bromide was used, which has mutagenic properties. When using the transluminator, it was necessary to have glasses with a UV filter and an acrylic plate between the equipment and the operator. Finally, the gel was incinerated in FAVET together with the gloves that were used for its handling.

Result

The detection of B. canis was performing the conventional PCR technique with positive and negative controls, the described primers and the specified protocol. A larger amplicons was obtained in the electrophoresis for DNA from B. canis, in relation to B. Sui and B. abortus. The (Figure 1) summarizes the results obtained in the implementation of this conventional PCR protocol. The sample of B. canis positive to the PCR implemented was sent in triplicate to the company Genetic and three sequences of around 430 bp were received. The use of the Crustal Ω multiple alignment programs allowed obtaining a representative sequence:

>VCL

CGAACACAAGGGCCAATACTCCCAATATGCG-TATCGCCAATCGGGTCCGTAATTGTCGGGCG-CCCACTTTCAACGTGCCGCATCTCCATTCACG-CAGACTTTATCTAGTCCATGATGGCCGTCTTTGC-CAAGGGACAAATAGTCCGGGGGGAGGAGCGAAT-TAAGGCAGTAAGGCAATAGGGCCATAAGGCAG-TATGTTAAGGGAATAGCGGAATAAGGGAGTAGGG-GAGTATGTATTTGGTTGCGCAAGGCCGCAGCGAC-CATATTCTTCACTGCCCTACTGCCCTACTGCCCTACT-GCCCTACTGCCCTACTGCCCTACTGCCCTACTG-CCCTACTGCCCTACTGCCCTACTGCCCTACTGC-CCTACTGCCCTACTGCCCTAAACATACCTCCCCGCGC-GCCT GAGCTCTTCGATCTTGTAGCC

Finally, the identity of the sequenced DNA fragment corresponded to B. Canis according to BLAST, with a Nucleotide Identity Percentage (NIP) of 97% (Figure 3).

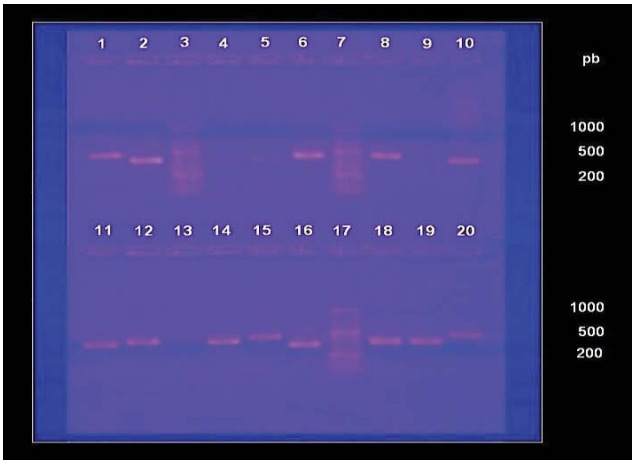

Figure 1: Differential detection of B. canis by conventional PCR. Electrophoresis in 3% agarose gel. Lanes 1, 6, 8, 15 and 20: B. canis; Lanes 2, 14 and 18: B. Sui; Lanes 3, 7 and 17: MTM; Lane 4: E. coli; Lane 5: S. Enteritidis; Lane 9: B. ovis; Lanes 10, 11 and 16: B. abortus C19; Lanes 12 and 19: B. abortus RB51; Lane 13: Control reagents. MTM: molecular size marker (50-1000 bp (Bio line®).

Discussion and conclusion

Our results demonstrating that the close nucleotide relationship between B. canis and B. suis [10] would not constitute an obstacle to establish a diagnosis molecular differential, considering a different experimental approach: to determine the existence of a non-conserved zone within the genome of B. canis and then the in silico design of appropriate starters for the implementation of a conventional PCR protocol.

Currently, the use of the PCR technique in the detection of bacteria of the genus Brucella does not imply any novelty,

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B.canis     TAAGGCAGTAAGGCAATAGGGCAATAAGGCAGTATGTTAAGGGAATAGGGGAATAAGGGA   62678
B.suis      TAAGGCAGTAAGGCAATAGGGCAATAAGGCAGTATGTTAAGGGAATAGGGGAATAAGGGA   62780
            ************************************************************

B.canis     GTAGGGGAGTATGTTTTTGGTTGCGCATGGCCGCAGCGACCATATTCTTCACTGCCCTAC   62738
B.suis      GTAGGGGAGTATGTTTTTGGTTGCGCATGGCCGCAGCGACCATATTCTTCACTGCCCTAC   62840
            ************************************************************

B.canis     TGCCCTACTGCCCTACTGCCCTACTGCCCTACTGCCCTACTGCCCTACTGCCCTACTGCC   62798
B.suis      TGCCCTACTGCCCTA---------------------------------------------   62900
            ***************

B.canis     CTACTGCCCTACTGCCCTACTGCCCTACTGCCCTAAACATACCTCCCCGCGC           62858
B.suis      -------------------CTGCCCTACTGCCCTACTGCCCTAAACATACCTCCCCGCGC   62960
                               ****************************************

B.canis     GCTGGAGCACTTCGATCTTGTAGCCGTCCGGGTCTTCCACGAAGAAGAAGCGGGCGAGGA   62918
B.suis      GCTGGAGCACTTCGATCTTGTAGCCGTCCGGGTCTTCCACGAAGAAGAAGCGGGCGAGGA   63020
            ************************************************************

B.canis     GAGCGCCGTTGTTCCTGAAATCCACCAGATTGCCGACCTTGAAGCCCAGTTCCCTGAAGC   62978
B.suis      GAGCGCCGTTGTTCCTGAAATCCACCAGATTGCCGACCTTGAAGCCCAGTTCCCTGAAGC   63080
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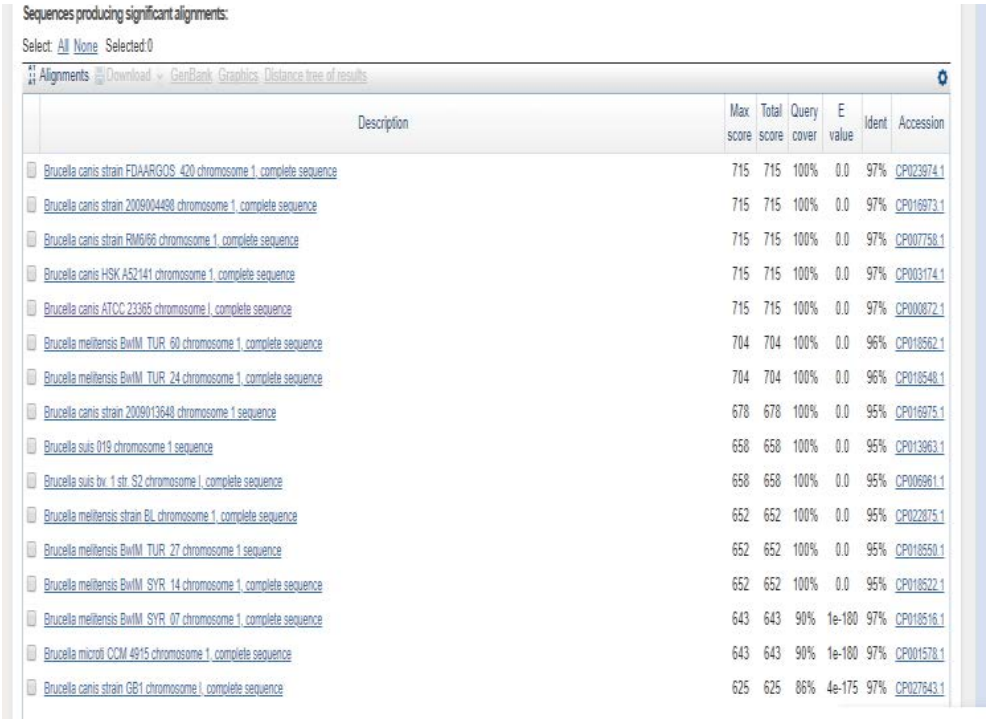
Figure 2: CLUSTAL Ω (1.2.4) Multiple sequence alignment. Show the unequal zone between B.canis and B.suis (64 bases minus)



Sequences producing significant alignments:

Select: All None Selected 0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Brucella canis strain FDAARGOS_420 chromosome 1, complete sequence	715	715	100%	0.0	97%	CP023974.1
Brucella canis strain 2009004498 chromosome 1, complete sequence	715	715	100%	0.0	97%	CP016973.1
Brucella canis strain RM6/66 chromosome 1, complete sequence	715	715	100%	0.0	97%	CP007758.1
Brucella canis HSK A52141 chromosome 1, complete sequence	715	715	100%	0.0	97%	CP003174.1
Brucella canis ATCC 23365 chromosome I, complete sequence	715	715	100%	0.0	97%	CP000872.1
Brucella melitensis BwIM_TUR_60 chromosome 1, complete sequence	704	704	100%	0.0	96%	CP018562.1
Brucella melitensis BwIM_TUR_24 chromosome 1, complete sequence	704	704	100%	0.0	96%	CP018548.1
Brucella canis strain 2009013648 chromosome 1 sequence	678	678	100%	0.0	95%	CP016975.1
Brucella suis 019 chromosome 1 sequence	658	658	100%	0.0	95%	CP013963.1
Brucella suis bv. 1 str. S2 chromosome I, complete sequence	658	658	100%	0.0	95%	CP006961.1
Brucella melitensis strain BL chromosome 1, complete sequence	652	652	100%	0.0	95%	CP022875.1
Brucella melitensis BwIM_TUR_27 chromosome 1 sequence	652	652	100%	0.0	95%	CP018550.1
Brucella melitensis BwIM_SYR_14 chromosome 1, complete sequence	652	652	100%	0.0	95%	CP018522.1
Brucella melitensis BwIM_SYR_07 chromosome 1, complete sequence	643	643	90%	1e-180	97%	CP018516.1
Brucella microti CCM 4915 chromosome 1, complete sequence	643	643	90%	1e-180	97%	CP001578.1
Brucella canis strain GB1 chromosome I, complete sequence	625	625	86%	4e-175	97%	CP027643.1

Figure 3: Sequences producing significant alignment with representative VCL sequence, according BLAST program (NIP=97%)

since in the literature it has been reported that the use of PCR has been successful in the diagnostic confirmation of B. canis [7, 8, 9], proving to be a sensitive, specific and rapid method for obtaining results [7], however, our results are novel when considering a differential molecular detection of these pathogens.

In this context, although the original idea that considers the high genomic homology presented by the bacterial genus had already been developed in recent studies, it was possible to establish a differential diagnosis of all the Brucella species existing to date, through the use of a PCR in its multiple version [12], B. canis was diagnosed by defect, that is, by the absence of an amplicons.

Instead, by the methodology described in our study, the diagnosis of B. canis is by effect, that is, by the differential presence of a determined amplicons, confirmed by nucleotide sequencing and by a genomic identity of around 97% with respect to GenBank. These results allow us to suggest the early implementation of this PCR as an alternative to bacterial isolation and therefore be confirmatory to serological tests, which would be very useful in moments prior to the crossing, avoiding erroneous diagnoses of animals that could be found, for example, infected by B. abortus or B. suis and not by B. canis, with the subsequent reproductive loss of the specimen. To the above, it should be added that this methodology would not constitute a risk to the laboratory staff, given that it works with DNA and not the viable agent.

Another reason why this method should be considered as alternative and complementary, is that currently in the diagnosis of Brucella the most used corresponds to serological tests since they have as main disadvantage both the obtaining of false positives product of the cross-reactivity with species bacterial of different or the same genus [15, 4] as the obtaining of false negatives in chronic cases of infection, given the intermittence of bacteremia, needing confirmation through bacterial isolation [1].

In terms of the implementation of the technique, both the design of in silico primers and the verification of nucleotide identity by means of bioinformatics tools has demonstrated once again its utility in the implementation of a conventional PCR for the detection of pathogens of veterinary interest in our laboratory [13, 14] and in this case able -for the first time- to differentiate by effect (obtaining an amplicons of a certain size) B. canis from B. suis and B. abortus, these last two species although do not have a greater implication in the dog, could lead to an erroneous diagnosis of the animal with the consequent cessation of its reproductive activity and / or euthanasia of the animal. Finally, the advisable to implement this technique in practice would be to use agarose gel at 4%, in order to make more noticeable the difference between the bands, added to this incorporate a positive control (DNA of B. canis) and a negative control (DNA of B. abortus) to analyze them together with the suspicious sample.

Thus, the identification of a genomic zone not conserved within the genome of B. canis, allowed the in silico design of a pair of specific primers, which allowed the obtaining of amplicons of different sizes achieving a differential diagnosis between bacteria of the genus Brucella, corroborated by a PIN of 97%. The above, could be a valuable contribution to the diagnosis of canine Brucellosis, as an alternative method to bacterial isolation.

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