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Use of Polymerase Chain Reaction and Endonuclease Restriction *Bcc*I for the differential *Leptospira interrogans* detection

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Abstract: ***Leptospirosis is a world disease, affecting animals and humans, which is transmitted by all infected animal, having the rodents as the most recognized. Leptospira interrogans serovar canicola, Leptospira interrogans serovar icterohaemorragiae, Leptospira interrogans serovar pomona and Leptospira interrogans serovar grippotyphosa affect canines, pigs, horses and cattle. A differential diagnosis method of four Leptospira serovars is our objective, through the molecular technique of the Polymerase Chain Reaction (PCR) adding the action of the restriction enzyme BccI, and thus generate fragments of different size for the recognition of the gene encoding a lipoprotein membrane LipL32. As conclusion, the detection of Leptospira interrogans by PCR was possible by that the primers designed for the realization of the method are specific for this agent. The differentiation of the 4 serovars of Leptospira was successful because the restriction enzyme cleaves the double strand of DNA in the 4 serovars, delivering expected results.***

Keywords: Leptospira, PCR, restriction endonuclease, *Bcc*I, LipL32.

1. Introduction

Leptospirosis is probably the most disseminated zoonosis in the world (Adler 2014). The microorganism infects a large variety of mammals, both domestic and wild, and the picture usually presents an abrupt onset with fever, myalgia and headache. Although most cases are mild or moderate, the clinical course is complicated by renal failure, uveitis, pulmonary hemorrhage, respiratory distress, myocarditis, rhabdomyolysis and meningitis (Olmo *et al.,* 2014). Normally dogs are vaccinated against two of the serovars of Leptospira interrogans: *canicola* and *icterohaemorragiae*, but this does not ensure complete immunity. Thus, to make an early and appropriate therapeutic decision, veterinarians have differentiated leptospirosis from other diseases, such as piroplasmosis or parvoviral enteritis, whose clinical signs are like those of leptospirosis, but which require completely different treatments. At present, the presence of infection is determined by the microscopic agglutination test (MAT), however, its results are ambiguous since agglutination occurs both because of the same vaccination and as an infection (Andre-Fontaine *et al.*, 2015). *Leptospires* are Gram-negative, helical bacteria, belong to the genus Leptospira, family *Leptospiraceae*, *spirochetal* order (Adler and de la Peña Moctezuma, 2010). DNA studies, determined its taxonomic classification. This is how the genus Leptospira includes three non-pathogenic species: *L. biflexa, L. meyerii, L. wolbachii, and seven pathogenic species: L. borgpetersenii, L. inadai, L. interrogans, L. kirschneri, L. noguchii, L. santarosai and L. weilii*; that contemplate 24 serogroups and 237 serovars (Zunino and Pizarro, 2007). A hook-shaped end is characteristic in *Leptospires*. It has two flagella, which are responsible for the movement of the spirochete (Adler and de la Peña Moctezuma, 2010). The genome of *L. interrogans*, consists of 4,691,184 base pairs (bp). It consists of two circular chromosomes, a large one of 4,332,241 bp and a smaller one of approximately 358,943 bp (Xiren *et al.*, 2003).

The Polymerase Chain Reaction (PCR) is an efficient molecular technique to achieve the diagnosis of leptospirosis in a fast, sensitive and specific way. This method has been used above all for the diagnosis of microorganisms difficult to grow and this is how several researchers have used it to detect serovars of *Leptospira* in the different clinical cases in which infection with this agent is suspected. In this context, in the present report it is proposed to use the PCR technique using as detection target the *Lip*L32 protein gene, a lipoprotein expressed in the outer membrane of the bacterium and highly conserved among the pathogenic species of Leptospira spp. (Cullen *et al*, 2002) in association with enzymatic digestion by means endonucleaseas a molecular method that allows to discriminate between *Leptospira interrogans* serovar *canicola,L. interrogans* serovar *pomona, L. interrogans* serovar *icterohaemorragiae* and *L. interrogans* serovar *grippotyphosa*, contributing to the epidemiological study of the disease (Jung *et al*.,2015).

The main difference between with previous studies is the differentiation between the 4 serovars of *L. interrogans*, through the action of a restriction endonuclease *Bcc*I. The selection of this enzyme was carried out thanks to the free access program Neb cutter ™. It was sought that this 480 bp amplicon was digested by the restriction enzyme BccIto differentiate between *L. interrogans* serovar *canicola* (LIC; AJ580493.1), *L. interrogans* serovar *Pomona* (LIP; EU871716.1), *L. interrogans* serovar Icterohaemorragiae (LII; AB094433.2) and *L. interrogans* serovar grippotyphosa (LIG; JN886738.1).
It should be noted that the restriction enzyme cleaved double-stranded DNA concomitantly with the detection of a specific base sequence. Thus, the BccI enzyme -isolated from *Bacteroides caccae*- recognizes the nucleotide sequence: CCATCNNNNNN (Genbank, 2015).

2. Materials and Methods

The work was carried out in the Virology and Microbiology Laboratories of the Department of Preventive Animal Medicine, University of Chile and in the Laboratory of Leptospira of the Agricultural and Livestock Service of Chile (SAG).

Implement the conventional PCR protocol: Pure strains obtained from cultures of the Leptospirosis unit of the bacteriology laboratory of SAG were used: the following serovars: *Leptospira interrogans* serovar canicola (LIC), *Leptospira interrogans* serovar *icterohaemorragiae* (LII), *Leptospira interrogans* serovar *pomona* (LIP) and *Leptospira interrogans* serovar grippotyphosa (LIG). As a negative control, *Salmonella* Typhimorium DNA was used. Nucleate-free water (NFW) was used as reagent control.

2.1 Obtaining DNA from Leptospira

DNA extraction was carried out using a commercial kit (High pure PCR template preparation kit Roche

2.2. Primers

In the PCR technique, the primers JB1 were used: 5'-ATGTTTGGATTCCTGCCGTA-3 'and JB2: 5'-GGCTC ACACCTGGAATACCT-3' generated by the Oligoperfect Design program (Invitrogen, ltd.) for free *online* access to obtain an amplicon of 480 base pairs. The design of the primers was based on the alignment of the four serovars and the choice of the genomic zone that involves the nucleotide differences found according to the *Clustal* Omega free *online* program (Annexes 1 and 2). The reconstitution of each of the primers with nuclease-free water, led to the final obtaining of both primers at a concentration of 1 µM.

2.3 Reaction mixture

15 µL of the Master Mix Go taq® Green 2x PROMEGA M712b solution, 5 µL of the template DNA and 5 µL of each specific primer was used, reaching a final volume of 30 µL.

2.4 DNA amplification

The samples were taken to the Apollo® thermocycler and subjected to a DNA denaturation step at 94° C for 30 seconds, a stage of alignment of the starters at 55° C for 30 seconds and an elongation stage at 72° C for 30 seconds, to complete a cycle, requiring 30 cycles to perform the method. After 30 cycles, the amplified product was visualized.

2.5 Visualization of the amplified product

It was carried out by electrophoresis in 2% agarose gel (Winkler ®) and was carried out at 90 V for 90 minutes. As a molecular size marker, AccuRuler 100 bp Ladder Maestrogen® was used, ranging from 100 to 3000 base pairs. The gel was incubated in ethidium bromide (0.5 µg/mL) (Fermelo ®) for 40 minutes and then placed in a ultraviolet light Transiluminator UVP ®. Finally, the gel was photographed to obtain a record of the results.

2.6 Obtaining a typical digestion pattern for each serovar of *Leptospira interrogans*

Each DNA fragment obtained in PCR was digested with the restriction enzyme *Bcc*I. To start the digestion, the 4 tubes were placed in an oven at 37° C for 1 hour and 20 minutes, and to inactivate the enzyme, these tubes were taken to a 65° C bath for 20 minutes. The visualization of the digested and undigested fragments was carried out in the same way as mentioned above.

2.7 Biosafety regulations.

Use of clean and closed aprons, use of clean material and proper disposal of waste. Regarding the PCR procedure and subsequent electrophoresis, latex gloves should be used both to avoid contamination of the sample and to manipulate substances such as ethidium bromide, which has mutagenic properties. When using the transilluminator, glasses with UV filter and an acrylic plate located between the equipment and the person who visualized the gel were used.

3. Results

3.1 Detection by PCR of the *lip*L32 gene in strains of *L. interrogans*.

The visualization of amplicons by the PCR technique with the designed *in silico* primers show in all the samples a single band around 500 bp. No bands were observed in the lanes assigned to the negative control or the reagent control. (Figure 1).

3.2 Determination of the utility of the *Bcc*I enzyme in *L. interrogans* to differentiate between serovars *pomona, icterohaemorragiae, canicola* and *grippotyphosa* of *L. interrogans.*

After the digestion of the DNA fragments products of the PCR technique used, several fragments of different sizes are observed in each lane, according to Figure 2.

4. Discussion

The PCR protocol implemented with the primers designed *in silico*, was able to detect unique fragments of the expected size (≈500 bp), which is certainly a contribution to the general detection of Leptospira. In addition, when carrying out the enzymatic digestion with *Bcc*I, the expected pattern was obtained, since single bands of around 480 to 70 bp were observed in each of the digested fragments and PCR products (Anexx 4).

These results were expected considering the cut-off sites of the acquired restriction enzyme. Theoretically, the restriction enzyme would generate a differential profile according to each serovar. Thus, by observing the electrophoretic pattern, the utility of endonuclease use can be clearly established.

The use of restriction enzymes in differential detection between serovars of Leptospira has been reported recently, but using 2 or more restriction enzymes such as *Bas*JI, *Fok*I, *Hae*III, *Hyp8*I and *Taq*II for the digestion of amplicons generated by PCR (Wajjwalku *et al.*, 2015).

This represents an important difference with our work in terms of using two or more enzymes for the differentiation of serovars.

Currently, other genes have been described as targets for detection of Leptospira: the *flab* gene (Wajjwalku *et al.*,

2015) or the β-subunit gene of RNA polymerase (Jung *et al.*, 2015), which increases the number of targets for detection by PCR and subsequent digestion with one, or with several restriction enzymes. However, it has been described that the *lip*l32 gene is the most conserved in the pathogenic species of Leptospira, which makes it suppose that it has an infective character, even though its role is not absolutely clear (Murray 2013).

In summary, the *Bcc*I constitutes a great choice for the differentiation of *Leptospira* serovars.

5. Conclusion

Without a doubt, the molecular detection of *Leptospira interrogans* is a reality, since it is demonstrated with the use of the PCR protocol already described. There is no debt in the differential detection of serovars, which according to the literature and the use of the *online* program NEB cutter allowed to obtain a very encouraging result with the use of *Bcc*I.

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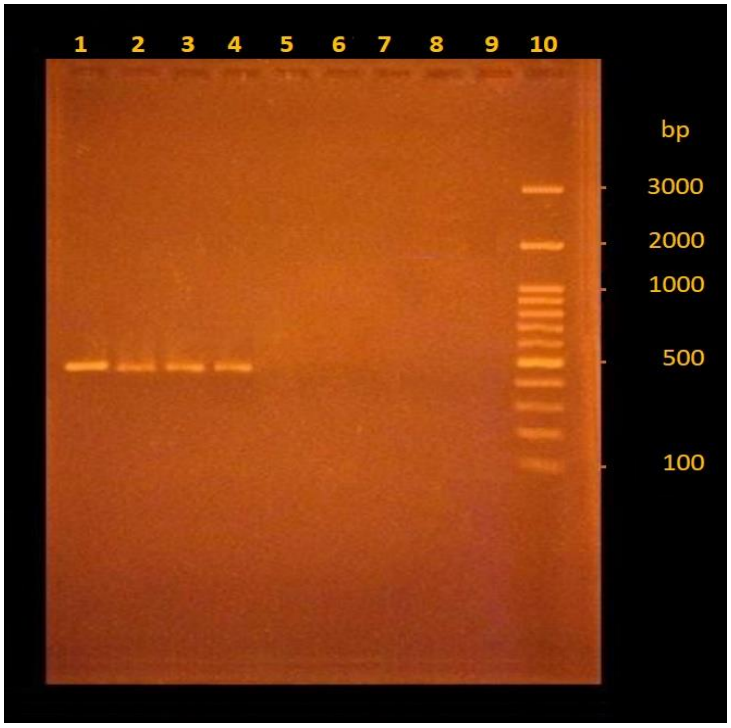

Figure 1
Agarose gel electrophoresis of the *lip*L32 gene for *Leptospira interrogans*.
Lane 1: LIC. Lane 2: LII. Lane 3: LIP. Lane 4: Lane 5: negative control.
Lane 6: control of reagents. Lanes 7, 8.9: Empty. Lane 10: molecular size marker

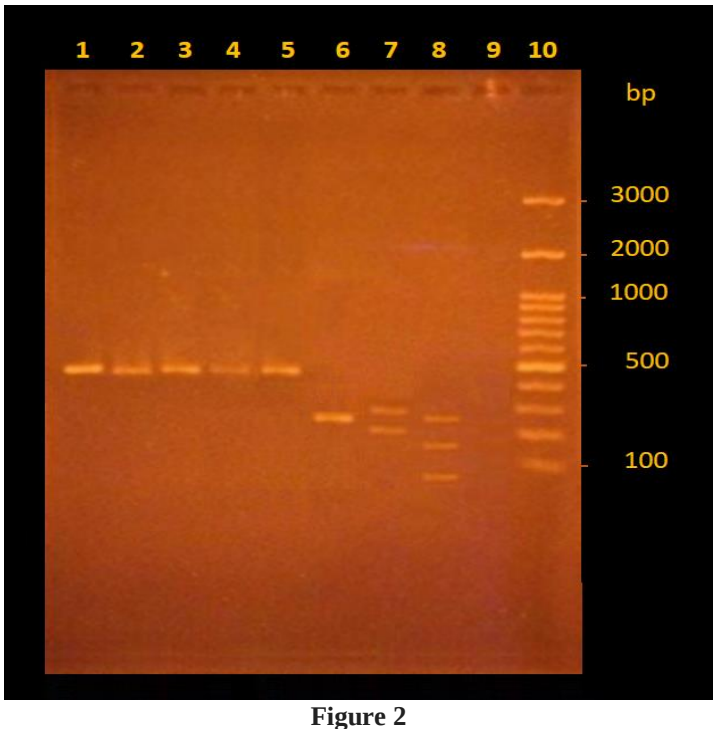

Figure 2
Agarose gel electrophoresis. Digestion with *Bcc*I for *Leptospira interrogans*.
Lane 1: LIC (PCR). Lane 2: LIP (PCR). Lane 3: LII (PCR). Lane 4: LIG (PCR).
Lane 5: LIC (restriction). Lane 6: LIP (restriction). Lane 7: LII (restriction).
Lane 8: LIG (restriction). Lane 9 empty. Lane 10: molecular size marker

Anexx 1: Nucleotide sequences from *Leptospira interrogans* serovars.

>AJ580493: *Leptospira interrogans* serovar *canicola*: LIC
ATGAAAAAACTTTCGATTTTGGCTATCTCCGTTGCACTCTTTGCAAGCATTACCGCTTGTGGTGCTTTCGGTGGTCTG
CCAAGCCTAAAAAGCTCTTTTGTTCTGAGCGAGGACACAATCCCAGGGACAAACGAAACCGTAAAAACGTTACTCCCT
ACGGATCTGTGATCAACTATTACGGATACGTAAAGCCAGGACAAGCGCCGGACGGTTTAGTCGATGGAAACAAAAAAG
CATACTATCTCTATGTTTGGATTCCTGCCGTAATCGCTGAAATGGGAGTTCGTATGATTTCCCCAACAGGCGAAATCG
GTGAACCAGGCGACGGAGACTTAGTAAGCGACGCTTTCAAAGCGGCTACCCCAGAAGAAAAATCAATGCCACATTGGT
TTGATACTTGGATCCGTGTAGAAAGAATGTCGGCGATTATGCCTGACCAAATCGCCAAAGCTGCGAAAGCAAAACCAG
TTCAAAAATTGGACGATGATGATAATGGTGACGATACTTATAAAGAAGAGAGACACAACAAGTACAACTCTCTTACTA
GAATCAAGATCCCTAATCCTCCAAAATCTTTTGACGATCTGAAAAACATCGACACTAAAAAACTTTTAGTAAGAGGTC
TTTACAGAATTTCTTTCACTACCTACAAACCAGGTGAAGTGAAAGGATCTTTCGTTGCATCTGTTGGTCTGCTTTTTC
CACCAGGTATTCCAGGTGTGAGCCCGCTGATCCACTCAAATCCTGAAGAATTGCAAAAACAAGCTATCGCTGCTGAAG
AGTCTTTGAAAAAAGCTGCTTCTGACGCGACTAAGTAA

>AB094433.2: *Leptospira interrogans* serovar *icterohaemorrhagiae*: LII
TTACCGCTTGTGGTGCTTTCGGTGGTCTGCCAAGCCTAAAAAGCTCTTTTGTTCTGAGCGAGGACACAATCCCAGGGA
CAAACGAAACCGTAAAAACGTTACTTCCCTACGGATCTGTGATCAACTATTACGGATACGTAAAGCCAGGACAAGCGC
CGGACGGTTTAGTCGATGGAAACAAAAAAGCATACTATCTCTATGTTTGGATTCCTGCCGTAATCGCTGAAATGGGAG
TTCGTATGATTTCCCCAACAGGCGAAATCGGTGAGCCAGGCGACGGAGACTTAGTAAGCGACGCTTTCAAAGCGGCTA
CCCCAGAAGAAAAATCAATGCCACATTGGTTTGATACTTGGATCCGTGTAGAAAGAATGTCGGCGATTATGCCTGACC
AAATCGCCAAAGCTGCGAAAGCAAAACCAGTTCAAAAATTGGACGATGATGATGATGGTGACGATACTTATAAAGAAG
AGAGACACAACAAGTACAACTCTCTTACTAGAATCAAGATCCCTAATCCTCCAAAATCTTTTGACGATCTGAAAAACA
TCGACACTAAAAAACTTTTAGTAAGAGGTCTTTACAGAATTTCTTTCACTACCTACAAACCAGGTGAAGTGAAAGGAT
CTTTCGTTGCATCTGTTGGTCTGCTTTTCCCACCAGGTATTCCAGGTGTGAGCCCGCTGATCCACTCAAATCCTGAAG
AATTGCAAAAACAAGCTATCGCTGCT

>JN886738.1: *Leptospira interrogans* serovar *grippotyphosa*: LIG
ATGAAAAAACTTTCGATTTTGGCTATCTCCGTTGCACTCTTTGCAAGCATTACCGCTTGTGGTGCTTTCGGTGGTCTG
CCAAGCCTAAAAAGCTCTTTTGTTCTGAGCGAGGACACAATCCCAGGGACAAACGAAACCGTAAAAACGTTACTTCCC
TACGGATCTGTGATCAACTATTACGGATACGTAAAGCCAGGACAAGCGCCGGACGGTTTAGTCGATGGAAACAAAAAA
GCATACTATCTCTATGTTTGGATTCCTGCCGTAATCGCTGAAATGGGAGTTCGTATGATTTCCCCAACAGGCGAAATC
GGTGAACCAGGCGATGGAGACTTAGTAAGCGACGCTTTCAAAGCGGCTACCCCAGAAGAAAAATCAATGCCACATTGG
TTTGATACTTGGATCCGTGTAGAAAGAATGTCGGCGATTATGCCTGACCAAATCGCCAAAGCTGCGAAAGCAAAACCC
GTTCAAAAATTGGACGATGATGATGATGGTGACGATACTTATAAAGAAGAGAGACACAATAAGTACAACTCTCTTACT
AGAATCAAGATCCCTAATCCTCCAAAATCTTTTGACGACCTGAAAAACATCGATACTAAAAAACTTTTAGTAAGAGGT
CTTTACAGAATTTCTTTCACTACCTACAAACCAGGTGAAGTGAAAGGATCTTTCGTTGCATCTGTTGGTCTGCTTTTC
CCACCAGGTATTCCAGGTGTGAGCCCGCTGATCCACTCAAATCCTGAAGAATTGCAAAAACAAGCTATCGCTGCTGAA
GAGTCTTTGAAAAAAGCTGCTTCTGACGCGACTAAGTAA

>EU871716.1: *Leptospira interrogans* serovar *pomona:* LIP
ATGAAAAAACTTTCGATTTTGGCTATCTCCGTTGCACTCTTTGCAAGCATTACCGCTTGTGGTGCTTTCGGTGGTCTG
CCAAGCCTAAAAAGCTCTTTTGTTCTGAGCGAGGACACAATCCCAGGGACAAACGAAACCGTAAAAACGTTACTTCCC
TACGGATCTGTGATCAACTATTACGGATACGTAAAGCCAGGACAAGCGCCGGACGGTTTAGTCGATGGAAACAAAAAA
GCATACTATCTCTATGTTTGGATTCCTGCCGTAATCGCTGAAATGGGAGTTCGTATGATTTCCCCAACAGGCGAAATC
GGTGAACCAGGCGACGGAGACTTAGTAAGCGACGCTTTCAAAGCGGCTACCCCAGAAGAAAAATCAATGCCACATTGG
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GTTCAAAAATTGGACGATGATGATGATGGTGACGATACTTATAAAGAAGAGAGACACAACAAGTACAACTCTCTTACT
AGAATCAAGATCCCTAATCCTCCAAAATCTTTTGACGATCTGAAAAACATCGACACTAAAAAACTTTTAGTAAGAGGT
CTTTACAGAATTTCTTTCACTACCTACAAACCAGGTGAAGTGAAAGGATCTTTCGTTGCATCTGTTGGTCTGCTTTTC
CCACCAGGTATTCCAGGTGTGAGCCCGCTGATCCACTCAAATCCTGAAGAATTGCAAAAACAAGCTATCGCTGCTGAA
GAGTCTTTGAAAAAAGCTGCTTCTGACGCGACTAAGTAA

Anexx 2: Alignment of nucleotide sequences according Clustal Ω

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LIC    GGTGCTTTCGGTGGTCTGCCAAGCCTAAAAAGCTCTTTTGTTCTGAGCGAGGACACAATC    120
LIG    GGTGCTTTCGGTGGTCTGCCAAGCCTAAAAAGCTCTTTTGTTCTGAGCGAGGACACAATC    120
LII    GGTGCTTTCGGTGGTCTGCCAAGCCTAAAAAGCTCTTTTGTTCTGAGCGAGGACACAATC    71
LIP    GGTGCTTTCGGTGGTCTGCCAAGCCTAAAAAGCTCTTTTGTTCTGAGCGAGGACACAATC    120
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LIC    CCAGGGACAAACGAAACCGTAAAAACGTTAC-TCCCTACGGATCTGTGATCAACTATTAC    179
LIG    CCAGGGACAAACGAAACCGTAAAAACGTTACTTCCCTACGGATCTGTGATCAACTATTAC    180
LII    CCAGGGACAAACGAAACCGTAAAAACGTTACTTCCCTACGGATCTGTGATCAACTATTAC    131
LIP    CCAGGGACAAACGAAACCGTAAAAACGTTACTTCCCTACGGATCTGTGATCAACTATTAC    180
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LIC    GGATACGTAAAGCCAGGACAAGCGCCGGACGGTTTAGTCGATGGAAACAAAAAAGCATAC    239
LIG    GGATACGTAAAGCCAGGACAAGCGCCGGACGGTTTAGTCGATGGAAACAAAAAAGCATAC    240
LII    GGATACGTAAAGCCAGGACAAGCGCCGGACGGTTTAGTCGATGGAAACAAAAAAGCATAC    191
LIP    GGATACGTAAAGCCAGGACAAGCGCCGGACGGTTTAGTCGATGGAAACAAAAAAGCATAC    240
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LIC    TATCTCTATGTTTGGATTCCTGCCGTAATCGCTGAAATGGGAGTTCGTATGATTTCCCCA    299
LIG    TATCTCTATGTTTGGATTCCTGCCGTAATCGCTGAAATGGGAGTTCGTATGATTTCCCCA    300
LII    TATCTCTATGTTTGGATTCCTGCCGTAATCGCTGAAATGGGAGTTCGTATGATTTCCCCA    251
LIP    TATCTCTATGTTTGGATTCCTGCCGTAATCGCTGAAATGGGAGTTCGTATGATTTCCCCA    300
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LIC    ACAGGCGAAATCGGTGAACCAGGCGACGGAGACTTAGTAAGCGACGCTTTCAAAGCGGCT    359
LIG    ACAGGCGAAATCGGTGAACCAGGCGATGGAGACTTAGTAAGCGACGCTTTCAAAGCGGCT    360
LII    ACAGGCGAAATCGGTGAGCCAGGCGACGGAGACTTAGTAAGCGACGCTTTCAAAGCGGCT    311
LIP    ACAGGCGAAATCGGTGAACCAGGCGACGGAGACTTAGTAAGCGACGCTTTCAAAGCGGCT    360
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LIC    ACCCCAGAAGAAAAATCAATGCCACATTGGTTTGATACTTGGATCCGTGTAGAAAGAATG    419
LIG    ACCCCAGAAGAAAAATCAATGCCACATTGGTTTGATACTTGGATCCGTGTAGAAAGAATG    420
LII    ACCCCAGAAGAAAAATCAATGCCACATTGGTTTGATACTTGGATCCGTGTAGAAAGAATG    371
LIP    ACCCCAGAAGAAAAATCAATGCCACATTGGTTTGATACTTGGATCCGTGTAGAAAGAATG    420
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LIC    TCGGCGATTATGCCTGACCAAATCGCCAAAGCTGCGAAAGCAAAACCAGTTCAAAAATTG    479
LIG    TCGGCGATTATGCCTGACCAAATCGCCAAAGCTGCGAAAGCAAAACCCGTTCAAAAATTG    480
LII    TCGGCGATTATGCCTGACCAAATCGCCAAAGCTGCGAAAGCAAAACCAGTTCAAAAATTG    431
LIP    TCGGCGATTATGCCTGACCAAATCGTCAAAGCTGCGAAAGCAAAACCAGTTCAAAAATTG    480
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LIC    GACGATGATGATAATGGTGACGATACTTATAAAGAAGAGAGACACAACAAGTACAACTCT    539
LIG    GACGATGATGATGATGGTGACGATACTTATAAAGAAGAGAGACACAATAAGTACAACTCT    540
LII    GACGATGATGATGATGGTGACGATACTTATAAAGAAGAGAGACACAACAAGTACAACTCT    491
LIP    GACGATGATGATGATGGTGACGATACTTATAAAGAAGAGAGACACAACAAGTACAACTCT    540
       ************ ************************************ ************

LIC    CTTACTAGAATCAAGATCCCTAATCCTCCAAAATCTTTTGACGATCTGAAAAACATCGAC    599
LIG    CTTACTAGAATCAAGATCCCTAATCCTCCAAAATCTTTTGACGACCTGAAAAACATCGAT    600
LII    CTTACTAGAATCAAGATCCCTAATCCTCCAAAATCTTTTGACGATCTGAAAAACATCGAC    551
LIP    CTTACTAGAATCAAGATCCCTAATCCTCCAAAATCTTTTGACGATCTGAAAAACATCGAC    600
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LIC    ACTAAAAAACTTTTAGTAAGAGGTCTTTACAGAATTTCTTTCACTACCTACAAACCAGGT    659
LIG    ACTAAAAAACTTTTAGTAAGAGGTCTTTACAGAATTTCTTTCACTACCTACAAACCAGGT    660
LII    ACTAAAAAACTTTTAGTAAGAGGTCTTTACAGAATTTCTTTCACTACCTACAAACCAGGT    611
LIP    ACTAAAAAACTTTTAGTAAGAGGTCTTTACAGAATTTCTTTCACTACCTACAAACCAGGT    660
       ************************************************************

LIC    GAAGTGAAAGGATCTTTCGTTGCATCTGTTGGTCTGCTTTTTCCACCAGGTATTCCAGGT    719
LIG    GAAGTGAAAGGATCTTTCGTTGCATCTGTTGGTCTGCTTTTCCCACCAGGTATTCCAGGT    720
LII    GAAGTGAAAGGATCTTTCGTTGCATCTGTTGGTCTGCTTTTCCCACCAGGTATTCCAGGT    671
LIP    GAAGTGAAAGGATCTTTCGTTGCATCTGTTGGTCTGCTTTTCCCACCAGGTATTCCAGGT    720
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LIC    GTGAGCCCGCTGATCCACTCAAATCCTGAAGAATTGCAAAAACAAGCTATCGCTGCTGAA    779
LIG    GTGAGCCCGCTGATCCACTCAAATCCTGAAGAATTGCAAAAACAAGCTATCGCTGCTGAA    780
LII    GTGAGCCCGCTGATCCACTCAAATCCTGAAGAATTGCAAAAACAAGCTATCGCTGCT---    728
LIP    GTGAGCCCGCTGATCCACTCAAATCCTGAAGAATTGCAAAAACAAGCTATCGCTGCTGAA    780
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>Candidate sequence for primer design (consensus)

GAAACCGTAAAAACGTTACTTCCCTACGGATCTGTGATCAACTATTACGGATACGTAAAGCCAGGACAAGCGCCGG
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ACCAAATCGCCAAAGCTGCGAAAGCAAAACCAGTTCAAAAATTGGACGATGATGATGATGGTGACGATACTTATAA
AGAAGAGAGACACAACAAGTACAACTCTCTTACTAGAATCAAGATCCCTAATCCTCCAAAATCTTTTGACGATCTG
AAAAACATCGACACTAAAAAACTTTTAGTAAGAGGTCTTTACAGAATTTCTTTCACTACCTACAAACCAGGTGAAG
TGAAAGGATCTTTCGTTGCATCTGTTGGTCTGCTTTTCCCACCAGGTATTCCAGGTGTGAGCCCGCTGATCCACTC
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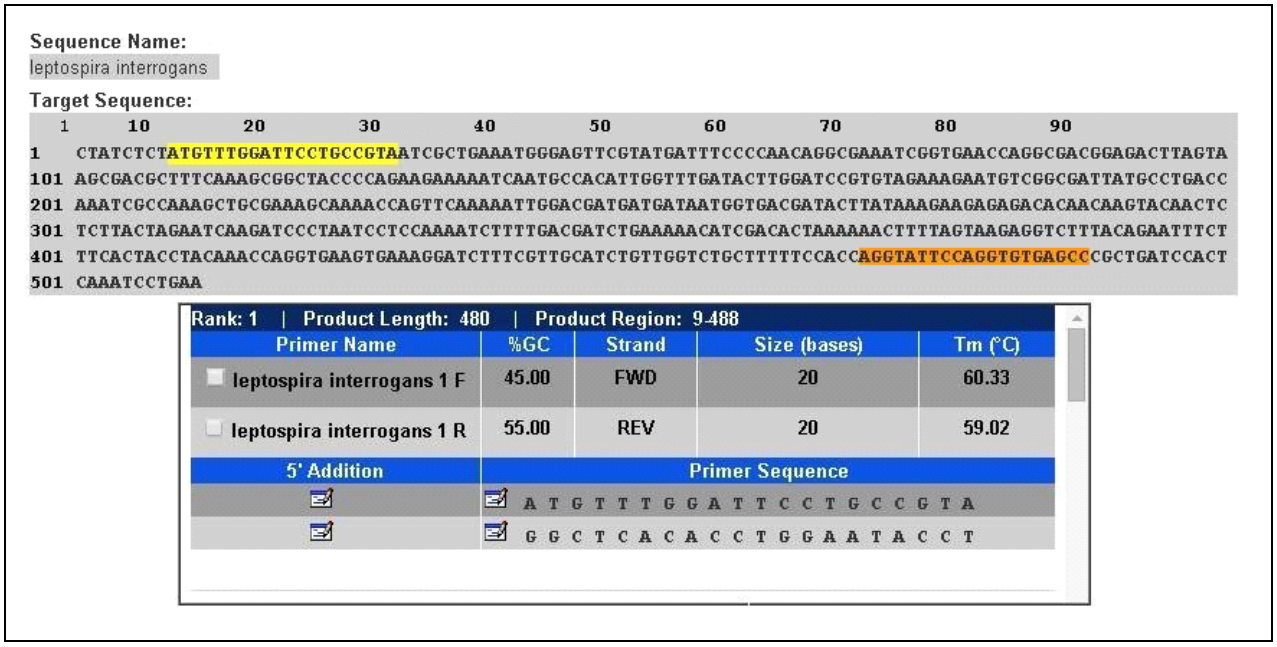
Anexx 3: *In silico* primer design (Invitrogen™Oliperfectdesign™)



Aneex 4: Custom digest with *Bcc*I (Bio Labs, NEBcutter).