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Comparison of different *Brucella* DNA markers as genus-specific PCR targets to improve detection sensitivity

By

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The undersigned hereby certify that they have read and recommend to the Faculty of Scientific Research and Higher Studies at the Palestine Polytechnic University and the Faculty of Science at Bethlehem University for acceptance a thesis entitled:

Comparison of different *Brucella* DNA markers as genus-specific PCR targets to improve detection sensitivity

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in biotechnology

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By: Abdelhalim Abu Awad Shahin

ABSTRACT

Brucellosis is a disease that causes serious problems for animals and human in many regions of the world, particularly, developing countries. The causative agent of brucellosis is the pathogenic bacteria called *Brucella*. The molecular techniques especially PCR considered as a standard method to detect *Brucella* because of their sensitivity. However, most primers for the detection of *Brucella* exhibiting variable sensitivity. Therefore, there is a need to design novel primers that target multi-sequences to obtain higher sensitivity than other existing primers used for *Brucella* detection. In this study, the de novo repeats finder tool softberry was used to mine for repetitive elements from the referenced genome (*Brucella melitensis bv. 1 str. 16M*) sequences. Two PCR reactions using IS1 and IS2 sets of primers were performed to assess their sensitivity in detecting *Brucella*. The designed short IS1 and long IS2 pair of primers were used to detect the insertion sequence IS711. The bands observed on agarose gel electrophoresis exhibited similar sensitivity of both B4-B5 and IS2 primers. However, IS1 primers possess lower sensitivity. By analyzing the structures of the targets using mfold online software, the IS711 targeted sequence has a complex secondary structure with a strong stem-loop hairpin structure that leads to obstacle the binding of the primer to its binding site and may lower the sensitivity of the PCR. We conclude that the secondary structure of the target at annealing step plays a critical role in the binding of the primers to the desired binding sites, affecting their sensitivity. We highly recommend analyzing the secondary structure of the selected target as an important criterion in designing a PCR reaction.

ملخص

الحمى المالطية هي مرض يسبب مشاكل جدية في الحيوان والانسان في مناطق مختلفة من العالم، وخاصة في الدول النامية. إن المسبب الممرض للحمى المالطية هي بكتيريا تدعى البروسيلا. إن التقنيات الوراثية التشخيصية خاصة البلمرة منها تعتبر من التقنيات الأساسية لفحص البروسيلا نظراً لدقتها. ولكن معظم المبلمرات المستخدمة تمتلك حساسية مختلفة بين بعضها البعض. لذلك، وجدت الحاجة لتطوير مبلمرات جديدة تستهدف مناطق متكررة في الجينوم والتي تظهر حساسية أعلى من الموجودة حالياً للكشف عن بكتيريا البروسيلا. في هذه الدراسة تم استخدام برنامج خاص للبحث عن السلاسل المتكررة وتم الكشف عن سلاسل متكررة خاصة بجينوم البروسيلا التي تصيب الاغنام. استخدم زوجان من المبلمرات من أجل عمل تقنية البلمرة وتقييم دقة الكشف عن بكتيريا البروسيلا. زوجا المبلمرات القصيرة IS1 والكبيرة IS2 - والتي تم تصميمها في هذه الدراسة – استخدمت من اجل الكشف عن القطعة الجينية المتحركة IS711. إن القطع التي تم ملاحظتها باستخدام تقنية فصل الآجار بالشحنات الكهربائية توضح التشابه ما بين حساسية الفحص بين كل من المبلمرات B4-B5 و IS2. ولكن المبلمرات IS1 لم تعطي حساسية مشابهة وانما أقل من ازواج المبلمرات السابقة. بناء على ذلك، تم فحص الشكل الجزيئي للسلاسل الجينية باستخدام برنامج الحاسوب mfold ووجد بأن مناطق ارتباط المبلمرات على السلاسل الجينية تحتوي على شكل مشبكيّ معقد والتي تؤدي الى عرقلة ارتباط المبلمر بالمنطقة المطلوبة. الأمر الذي يقلل من حساسية تقنية البلمرة. هنا نستطيع القول بأن الشكل الجزيئي للسلاسل الجينية المرغوب الكشف عنها يلعب دوراً مهماً في ارتباطهما في مرحلة الاتصال داخل تقنية البلمرة وبالتالي تؤثر على مدى حساسية الفحص. اننا ننصح وبشدة تحليل الشكل الجزيئي للسلاسل المستهدفة في عملية البلمرة قبيل البدء في تصميم المبلمرات الخاصة لذلك.

DECLARATION

I declare that the master thesis entitled "Comparison of different *Brucella* DNA markers as genus-specific PCR targets to improve detection sensitivity" is my own original work, and thereby certify that unless stated, all work contained within this thesis is my own independent research and has not been submitted for the award of any other degree at any institution, except where due acknowledgment is made in the text.

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DEDICATION

I would like to dedicate this work to my loving parents Amal and Sharif for their continuous support, without whom none of my success would be possible.

I would like to dedicate this thesis to my sisters and brothers for their support throughout my life.

For my lovely wife Assalla, I am truly grateful for having you in my life.

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ABBREVIATIONS

Abbreviation	Description
ΔG	Gibbs free energy change
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
dNTP	Deoxyribonucleoside triphosphate
DW	Distilled water
EDTA	Ethylenediaminetetraacetic acid
IS1	Short designed Primer
IS2	Long designed Primer
ISF1	Forward short designed Primer
ISR1	Reverse short designed Primer
ISF2	Forward long designed Primer
ISR2	Reverse long designed Primer
IS711	Insertion Sequence 711
L	Liter
MgSO4	Magnesium sulfate
Min	Minute
Ml	Milliliter
Ml	Microliter
Ng	Nanogram
NTC	No template control
PCR	Polymerase Chain Reaction
TE	Tris/EDTA
Tm	Melting temperature
DMSO	Dimethyl sulfoxide

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1. INTRODUCTION

1.1 Brucellosis: an Overview

Brucellosis is a bacterial zoonotic disease that affects many animals and humans. The causative agent of brucellosis is the pathogenic bacteria called *Brucella*. Dr. David Bruce isolated the first species (*Micrococcus Melitensis*) in 1887 from a British soldier spleen, and later the bacteria was renamed *Brucella melitensis* for his honor. In 1905, Dr. Themistocles Zammit identified the source of infection in human when he isolated *Brucella melitensis* from ovine milk (Godfroid et al., 2005). Until now, brucellosis is still considered one of the commonest zoonotic diseases worldwide (Seleem et al., 2010).

The disease causes serious financial burden and public health problems in many countries, particularly in developing countries. Various types of domestic animals are affected by *Brucella*, including cattle, sheep, goats, and camels. In these animals, the infection can cause reproductive problems such as abortion, infertility and reduction in milk production. The pathogen can be transmitted from infected animals to human via the ingestion of the unpasteurized milk and its products or by direct contact with infected animals or their contaminated biological materials (Khan, M et al., 2018, McDermott, J et al., 2013).

1.2 Epidemiology

Brucellosis is a global zoonotic disease with 500,000 new cases per year in human (Corbel, 2006). As shown in figure 1 and 2. The differences in the incidence rates depend on the animal health programs which differ from countries. According to the European Centre for Disease Prevention and Control (ECDC), the incidence rate in Europe in 2014 was 0.1 per 100,000 population. However, developing countries in North Africa, sub-Saharan Africa, Latin America, and Asia have a high incidence of brucellosis (Dean et al., 2012). The Middle East has the highest incidence rate particularly in regions with nomadic societies that rely on razing goats, sheep, and camels (Musallam et al., 2016). According to Dean et al., the highest incidence rate per 100,000 population were recorded in Iraq 268, Saudi Arabia 149 and Jordan 130 (Dean et al., 2012). Good animal health programs led to incidence rates close to reaching zero in countries such as Australia, Canada and numerous countries in northern Europe. However, the huge fluctuation in incidence rate in most developing countries is a result of

poor animal health programs and inefficient surveillance system (Hull et al., 2018).

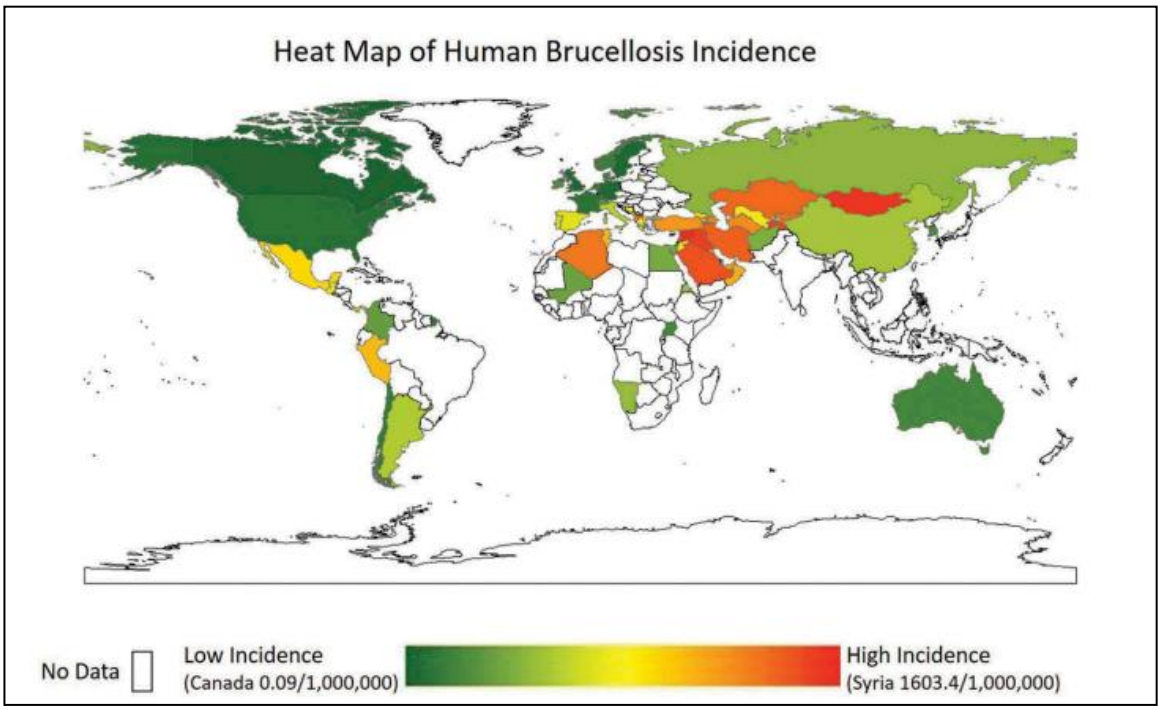


Figure 1.1: Human incidence rate per 1 million population

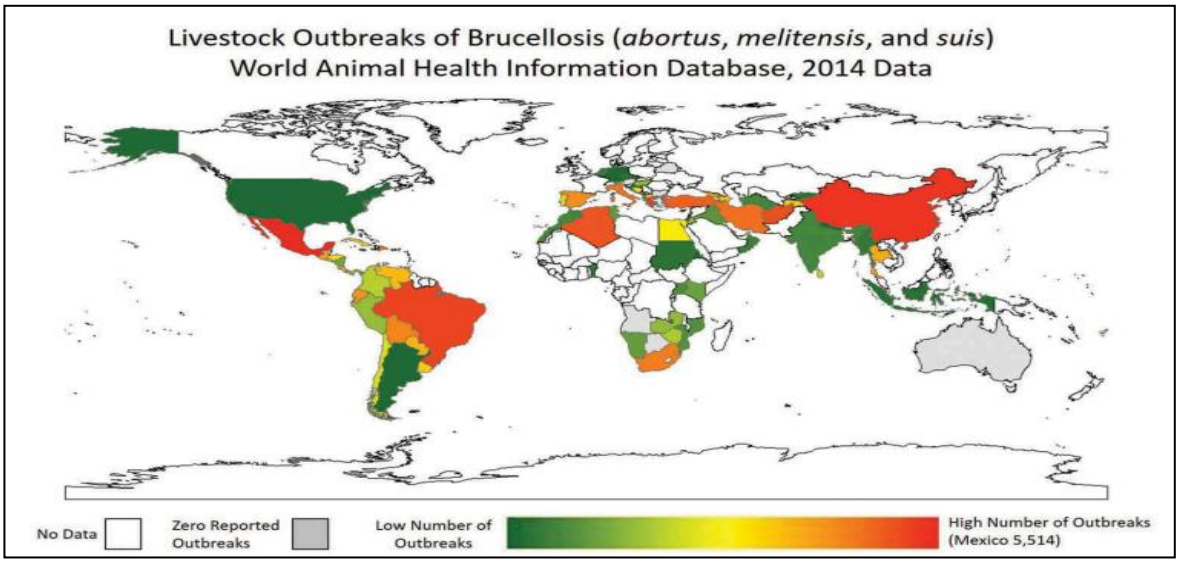


Figure 1.2: Livestock Outbreaks of Brucellosis

1.3 Clinical manifestations

The general symptoms of human brucellosis are fever, chill, fatigue, pyrexia, myalgia, arthralgia, and back pain. The possibility of developing complications in patients with Brucellosis is not rare. These complications could be endocarditis and neurological infection (Dean et al., 2012b). In rare cases, death was reported in some patients with severe endocarditis (Buzgan et al., 2010).

In animals, the symptoms vary from severe acute to sub-acute or chronic. These symptoms depend on the host specificity interaction. The common symptoms are pyrexia, carpel hygromas and retained placenta. The majority of animal losses and reduction of milk yield results from spontaneous abortion and mastitis which are typical features of brucellosis (Hall et al., 2018).

1.4 *Brucella* Genus

The causative agent of brucellosis in animals and human is *Brucella* genus. It belongs to the family *brucellaceae* of the order Rhizobiales. *Brucella* genus is a small, non-motile, gram-negative coccobacilli, aerobic, facultative intracellular bacteria that lack plasmid, spores and capsules (Banai et al., 2010). Taxonomic studies depending on DNA hybridization and sequencing of the 16s RNA gene techniques demonstrate a high degree of homology between species of this genus (VERGER et al., 1985). Currently, *Brucella* genus consists 12 species that are classified according to their pathogenicity and host preference. The species affect diverse types of wildlife and domestic mammals with a clear pattern of host preference. *B. abortus* mainly affect cattle, *B. melitensis* affect sheep and goats, *B. suis* affect pigs, while, *B. canis*, B. *ovis* and *B. neotomae* affect dogs, sheep and desert wood rats respectively. In marine mammals, *B. pinnipedialis* infects seals and sea lions, whereas *B. ceti* infects dolphin and whales. The pathogenic species that can cause brucellosis in human are; *B. meltenisis*, *B. abortus* . *B. canis and B.suis*. (El-Sayed et al., 2018)

1.5 Control regimens

1.5.1 Vaccination

Up to date, three vaccines were licensed for animal vaccination while there is no vaccine for the human. These are two smooth live vaccine strains B. abortus strain 19 for bovine, Rev1 for small ruminants and one rough strain RB51 for cattle. Even with the availability of these vaccines, a good vaccine for animal livestock is still a challenge for scientists. The currently used vaccines are live attenuated vaccines which are effective only in a specific host with potential for infection to the vaccinated animals (Schurig et al., 2002).

In Palestine, the vaccine used for sheep and goat vaccination program is *B. melitensis Rev.1*. It is the most effective vaccine against caprine and ovine brucellosis. However, several drawbacks have been reported, the efficiency is variable because of variation in production protocols, the vaccine strain can be shed in the milk of vaccinated animals for 1-2 months, and it may lead to infection in animals and in rare cases human (Banai et al., 2002).

1.5.2 Treatment

The treatment of brucellosis involves two antibiotic therapy for a total of six weeks. The treatment of choice includes either oral doxycycline and rifampicin for six weeks or a combination of 6 weeks of oral doxycycline and two or three weeks of intramuscular streptomycin. The latter treatment regimen using doxycycline and streptomycin is considered the gold standard because it is more effective. But, unfortunately, it is also more toxic and less convenient (Alavi et al., 2013). Mono-therapy and shorter course of treatment have been associated with higher relapse rates.

In complicated brucellosis infection such as spondylitis, neuro brucellosis, and endocarditis, the course of treatment is often extended to a minimum of twelve weeks and expanded to a third agent such as ceftriaxone or Bactrim (Liese et al., 2010). Practically, the mode of administration of the antibiotics is oral tablets for 6 weeks, and then, the disease will be monitored by serological tests. Monitoring the progress of treatment response is based on the measuring of IgG in the serum and the blood culture in at least 2 weeks' time interval (Ariza et al., 2007).

1.6 Diagnosis

1.6.1 Laboratory diagnosis

The variety of clinical manifestations of brucellosis results in clinical misdiagnosis of the disease leads to treatment failure. Therefore, an accurate, sensitive and specific diagnostic test must be confirmed in the lab. The main laboratory diagnostic tests used to detect *Brucella* relies on the serological tests RBT and STAT as the first-line tests for human brucellosis. Also, direct isolation of the bacteria by the cultural method is used for the detection of *Brucella* (Teng et al., 2017). The molecular techniques rely on the amplification and detection of *Brucella* DNA are widely used and have major advantages over other traditional tests in terms of sensitivity, specificity and time (Çiftci et al., 2017). Therefore, the molecular diagnostic techniques are essential for the laboratory tests used to detect *Brucella* pathogen

1.6.2 Serology

The Serological testing is widely used in the detection of *Brucella* especially in low-income countries. They are cost-effective, fast, safe and relatively sensitive. These tests are based on antibody-antigen reaction. The conventional serological tests used to detect brucellosis are Rose Bengal Test (RBT), Serum Tube Agglutination Test (STAT) and Complement Fixation Test (CFT). These tests detect antibodies against the smooth O-Chain Lipopolysaccharide (LPS). The possibility of cross-reactivity with other bacterial LPS results in lower specificity of the tests (Araj et al., 2010). Therefore, they must be combined with other tests like molecular techniques to confirm the diagnosis.

1.6.3 Bacterial Culture

Bacterial Culture is considered the gold standard test for laboratory diagnosis of brucellosis. It is based on culturing and isolating the bacterium in selective media (broth or solid). The bacterium in clinical specimens from different tissues or body fluids such as blood, CSF and bone marrow is inoculated in the media and incubated at 37˚C in 10% carbon dioxide atmosphere (Godfroid et al., 2010). The positive blood culture provides a definitive diagnosis. However, its time consuming, the bacterial growth is slow and usually takes 5 to 14 days. Additionally, it has poor sensitivity. The detection sensitivity varies from 15% to 70% (Araj et al., 2010). Also, the risk of infection for the lab workers is high.

1.6.4 Molecular detection

The molecular detection techniques based on DNA amplification becomes one of the standard methods in the detection of *Brucella* spp. It is specific, robust and safer than other assays and has the ability to detect even a small number of bacteria in clinical samples. These methods can identify the pathogenic agent in acute, subacute, and chronic infection in various types of clinical samples such as serum, blood and urine. Thus, the molecular methods proved its efficiency as an alternative tests rather than conventional assays for the diagnosis of brucellosis in clinical laboratories.

Many molecular assays have been developed for the detection of *Brucella*. The designed real-time RT-PCR protocols were proven to be more sensitive, specific, reproducible and rapid than conventional PCR and other serological and microbiological methods (Bounaadja et al., 2009). However, in low-

income countries such as Palestine, the technique is not common due to the high cost of the test. Another assay to detect *Brucella* is Loop-Mediated Isothermal Amplification Assay (LAMP). Unlike Polymerase Chain Reaction the reaction occurs at constant temperature (60˚C to 65˚C) with different sets of primers 4 or 6 primers targeting 6 or 8 regions within the genome (Lin et al., 2011). LAMP assay is a cheap technique that can be adopted in the developing countries. However, the complexity of primer design and non-specific products results from using a set of primes is still a major limitation of this technique. Al-Nakkas and his colleagues designed a single-tube nested PCR targeting the insertion sequence IS711 and tested it in a small number of samples, the sensitivity and specificity was 100% (Al Nakkas et al., 2002). Then, the assay was tested on larger samples while the result was 96.98% for the sensitivity and 100% for specificity (Al-Nakkas et al., 2005).

Many conventional PCR assays have been developed to detect *Brucella* spp. in different types of clinical samples (Al-Ajlan et al., 2011). These assays targeted different sequences at the genus and species level with variable values in sensitivity and specificity. This variability resulted from different extraction methods, clinical specimens and targeted sequences. Various primer sequences were designed to detect different genes existed in *Brucella* genome. For example, genes exist as a single copy in the genome such as the most used gene in the detection of bacterial agents is the 16S rRNA. Another single gene is the conserved gene encoding a 31 kDa immunogenic protein (BCSP31). Baily et al. designated B4 and B5 primer set that target the BCSP31 gene (Baily et al. 1992). These primers have been used as sensitive universal primers for the detection of *Brucella* in many labs. Other potential targets with more than one copy within the genome like the gene encoding an outer membrane protein antigen (omp-2) and the insertion sequence IS711 have also been used. However, even- though these targets exist in multiple copies; the results showed variable values in sensitivity and specificity (Al-Ajlan et al., 2011). Therefore, there is a great need to try to identify novel *Brucella* genus-specific markers that can improve detection sensitivity. In addition, to evaluate the detection sensitivity of different primer sets with an emphasis on the physicochemical properties of the targeted PCR template and not only focusing on the primers' physicochemical parameters.

2. OBJECTIVES

High sensitivity and specificity are achievable by molecular techniques such as polymerase chain reaction. However, most primers for the detection of *Brucella* exhibiting variable sensitivity. Although the target for B4-B5 primers (BCSP31) is one copy in *Brucella* genome, these primers are one of the most sensitive to detect *Brucella*. In order to improve the detection sensitivity, we assume that a genus-specific target with multiple copies can outperform the PCR targeting BCSP31. Therefore, this study aims to:

1. To identify a novel *Brucella* genus-specific and conserved target with repetitive nature.

2. To compare the performance in term of detection sensitivity of the newly designed primers with the B4-B5 primers.

3. To assess the influence of template secondary structure on the amplification efficiency.

3. MATERIALS AND METHODS

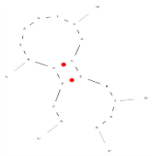
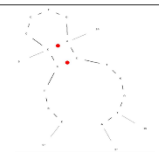
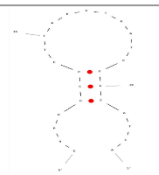
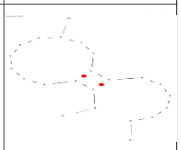
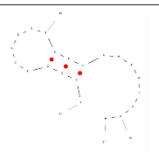
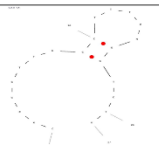
3.1 PCR Target Selection

The "*de novo* repeats finder tool" was downloaded via softberry website. This bioinformatic tool was used to mine for repetitive elements in the referenced genome *Brucella melitensis bv. 1 str. 16M*, which is $3.2x10^6$ bp. Only candidate sequences with ≥ 5 copies in the genome were screened. The Basic Local Alignment Search Tool (BLAST) was used to confirm genus specificity and conservation of the candidate sequences among all available completed *Brucella* genomes in the NCBI database. To determine the specificity of the marker, each candidate sequence was used as a query to perform Blastn against all available genomic sequences in the NCBI excluding *Brucella* genus using the default nucleotide collection database. To validate the existence of the repeat in all *Brucella* species, each candidate sequence was used as a query to search 88 complete *Brucella* genomes that were available as of August 2018 in NCBI database.

3.2 Primer Design

Two primer sets for *Brucella* detection were designed manually to target the identified repeat sequences using the OligoAnalyzer online tool (Table1). All primers were tested by OligoAnalyzer to ensure no self-dimers, hairpins, heterodimers structures with a Gibbs free energy (ΔG) below -6 Kcal/mol. The primers were synthesized by IDT (Integrated DNA Technologies) company (https://eu.idtdna.com/calc/analyzer). The expected product size is 222 bp for IS1 primers, 390 bp for IS2 primers and 224 bp for B4-B5 primers.

Table 3.1: The designed long, short and B4-B5 primers.

Primer name	Primer sequence	Length	G-C content	ΔG (kcal.mole^{-1}) for hairpin structure	The structure of primer hairpin
IS1 primers	F:GCGGACAGTCACCATAA	17 bp	52.9 %	0.02	
	R:CATGCCCTGGCTGATA	16 bp	56.2 %	-1.22	
IS2 primers	F:GAAGGCCCTTAAGTGATCGG CATCA	25 bp	52 %	-1.64	
	R:GCGATCTGCCTGCGACCTTC	20 bp	65 %	-0.46	
B4-B5 primers (Baily et al. 1992).	F:TGGCTCGGTTGCCAATA TCAA	21 bp	47.6 %	2.64-	
	R: CGCGCTTGCCTTTC AGGTCTG	21 bp	61.9 %	- 0.84	

3.3 DNA template

As a positive control template, a 1.34 ng/ µl DNA from the B. melitensis Rev-1 vaccine strain (JOVAC, Jordan, BCD0315) was used. In order to test the lower detection limit, this initial concentration was diluted by a 5- fold serial dilution to produce 7 DNA concentrations starting with 1.34 ng/ µl and ending with 85.7 fg/ µl. The concentration of the DNA was measured using Nanodrop implen (N50: 200-650 nm).

3.4 PCR Optimization

3.4.1 IS1 primers PCR

A Gradient PCR for the IS1 primers was performed to test different annealing temperatures (75°C-45°C). The PCR was performed in a 25 µl reaction containing 1× Taq Buffer, 2.5 mM $MgSO_4$, 0.2 mM each dNTP, 0.2 µM each inner primers, 0.1 µl of Taq DNA polymerase and 1 µl of DNA template. The PCR test was performed in MJ mini Bio-Rad PTC 1148 thermocycler (BioRad, USA). For cycling conditions: initial denaturation at 95°C for 5 minutes, and then 35 cycles of 94°C for 30 seconds, (75°C - 60°C) or (60°C - 45°C) for 30 seconds, and 72°C for 30 seconds. A volume of 20 µL of PCR amplicons were visualized by gel electrophoresis on a 1.5% agarose gel.

3.4.2 IS2 primers PCR

A Gradient PCR for the IS2 primers was performed. For optimization purposes, the range for annealing conditions was from 75°C to 48°C. The long primer reaction was prepared by mixing 1× Taq Buffer, 2.5 mM $MgSO_4$, 0.2 mM each dNTP, 0.2 µM each outer primers, 0.1 µl of Taq DNA polymerase and 1 µl of DNA template in a 25 µl reaction volume The reaction was carried out using MJ mini Bio-Rad PTC 1148 thermocycler (BioRad, USA). The Reactions was performed under the following cycling conditions: initial denaturation at 95°C for 5 minutes, and then 35 cycles of 94°C for 30 seconds, (75°C-60°C) or (60°C - 48°C) for 30 seconds, and 72°C for 30 seconds. A volume of 20 µL of PCR amplicons were visualized by gel electrophoresis on a 1.5% agarose gel.

3.4.3 B4-B5 Primers PCR

The PCR was performed in a 25 µl mixture containing 1× Taq Buffer, 2.5 mM $MgSO_4$, 0.2 mM each dNTP, 0.1 µl of Taq DNA polymerase and 1 µl of DNA template. The PCR cycling Conditions were as follows: initial denaturation at 95°C for 5 minutes, and then 40 cycles of 94°C for 40 seconds, 63°C for 30 seconds, and 72°C for 30 seconds and the final extension was 10 minutes at 72° C.

3.5 Detection of *Brucella* DNA

After selecting the optimal annealing temperature for IS1 and IS2 primer sets, three PCR experiments were performed for these two sets alongside the B4-B5 primer set. In each experiment, the 7 serial dilutions of DNA were tested in a duplicate format in parallel with no-template control (NTC). The mixture and PCR cycling conditions were as described in the previous sections.

3.6 Secondary structure analysis

In order to perform template secondary structure analysis, the sense strand of the three PCR amplicons were retrieved via Blast. For each template, the exact PCR amplicon size was tested. The mFold web server (Zuker et al., 2003) (DNA folding form) (http://unafold.rna.albany.edu/) were used for the prediction of the secondary structures of the three template sequences at temperature 95°C and at corresponding annealing temperature for each primer set (B4-B5 at 63°C, IS2 primers at 60°C and IS1 primers at 55°C).

3.7 Seconday structure inhibition

Two different approaches were attempted to inhibit DNA secondary structure formation of the template.

1. By increasing the denaturation temperature to 98˚C instead of 95˚C. 2. By using the secondary structure relaxing additive dimethyl sulfoxide (DMSO). Two different concentrations (2.5% and 5%) of the DMSO were added to PCR. The positive control was an insert of IS711, and its existence was confirmed in our laboratories (Diala, unpublished data).

4. RESULTS AND DISCUSSION

4.1 Target selection

To obtain a good target to improve DNA detection sensitivity and specificity for Brucella spp., DNA sequences fulfilling the following criteria were screened:

1- The target should exist exclusively in Brucella genus with no homologous sequence in any other bacteria or associated hosts.

2. The target sequence should be highly conserved among all species of the *Brucella* genus.

3. The target must exist in multiple conserved copies. The copy number of the target within the genome plays a role in the determination of the detection sensitivity of the assay. The abundance of target copy number increases the possibility of the primers to anneal to the target. Thus, the detection limit will be lower and the analytical sensitivity of the assay increases (Kalle et al., 2014). So, we decided to choose the repeat sequences as a target for the developed PCR reaction for their abundance.

The results obtained by the repeat finder tool showed 38 repeats with different length and copy number. Repeats with more than 300 bp were screened to obtain a good size that can be visualized by gel electrophoresis. Also, repeats with low variation within the sequence and with ≥5 copies in the genome were screened. Out of 38 repeats, the repeat fulfilling the above mentioned three criteria was the sequence in figure 4.1.

The results obtained from BLAST surprisingly showed that this marker is the insertion sequence (IS711) which is a not novel repeat. The insertion sequence IS711 is a mobile genetic element that exclusively exists in *Brucella* genus with multi-copy DNA sequences in all *Brucella* genomes (Mancilla et al., 2011). The complete length of IS711 is 842 bp. IS711 is encountered as conserved sequence upon all *Brucella* genome due to its low variation in the sequence at intra and inter-species level (Halling et al., 1993). Each *Brucella* species possess at least five copies of IS711, making it a good potential target for the diagnosis of *Brucella*.

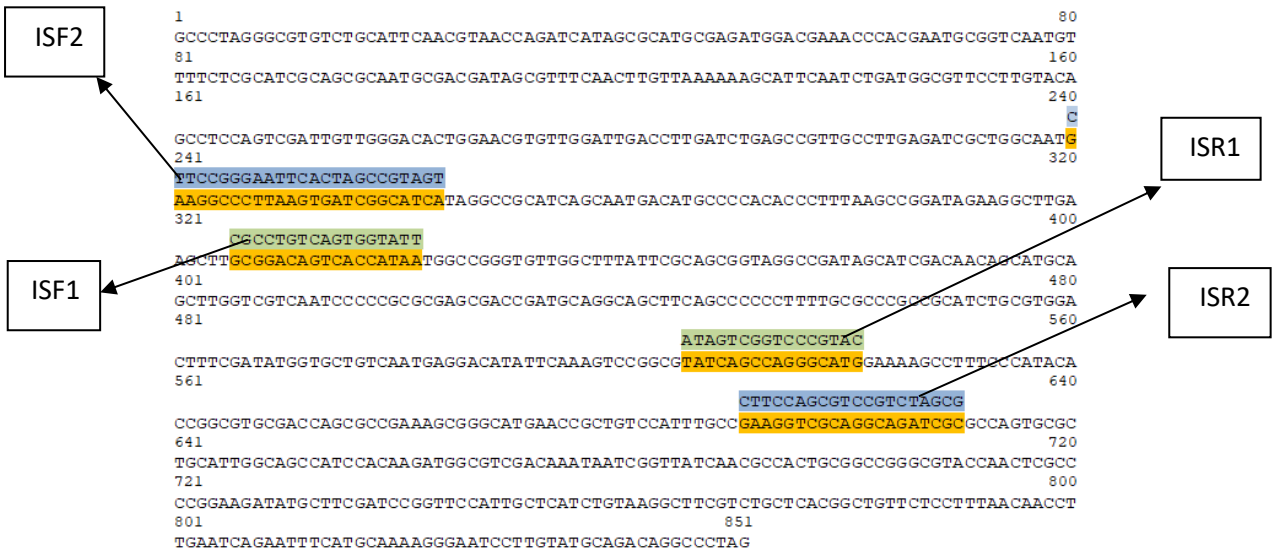


Figure 4.1: Targeted sequence IS711 with the location of the designed primers sets IS1 and IS2.

4.2 Gradient PCR for IS1 primers

To determine a good annealing temperature for IS1 primers, a gradient PCR was performed using B. melitensis Rev-1 vaccine strain DNA. The annealing temperatures range was between 75˚C to 45˚C. The results shown in figure 4.2 show that the IS1 primers are inactive at temperatures from 75˚C to 63.2˚C. While, at temperatures less than 61.1˚C to 45˚C, the inner primers are active. The temperature 55˚C was chosen as an optimal annealing temperature to perform detection sensitivity test.

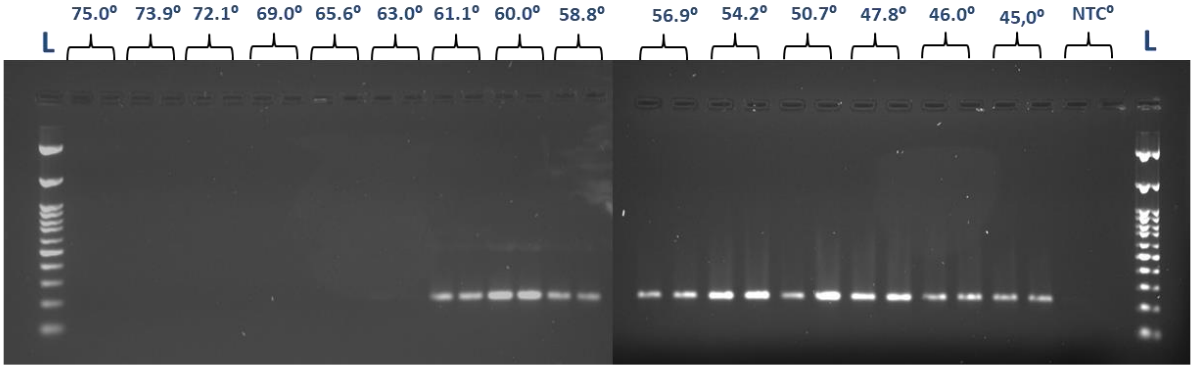


Figure 4.2: Agarose Gel Electrophoresis for the gradient PCR using the IS1 primers
L: 100 bp ladder.

4.3 Comparison of detection sensitivity for IS1 and B4-B5 primers

To compare the detection sensitivity of the IS1 and B4-B5 primers, the two sets were used to amplify a serial dilution of Rev-1 vaccine strain DNA. As shown in figure 4.4, the B4-B5 primers were able to detect the first 3 serial dilutions (1.34 ng/µl to 0.0536 ng/µl) with good band intensity. On the other hand, the fourth dilution (0.01072 ng/µl) was detected but with low-band intensity. However, the IS1 primers bands intensity is less than B4-B5 primers, the first two dilutions were detected with good band intensity, while the amplification results for the lower dilution were either very faint or negative (Figure 4.3).

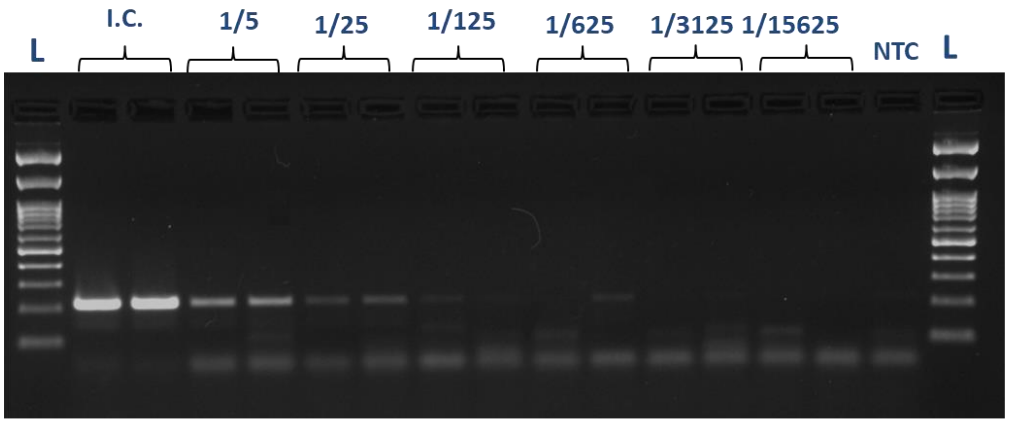


Figure 4.3: Agarose Gel Electrophoresis for the PCR using the IS1 primers.
L: 100 bp ladder., I.C.: Initial Concentration: 1.34 ng/µl, NTC: no template control.

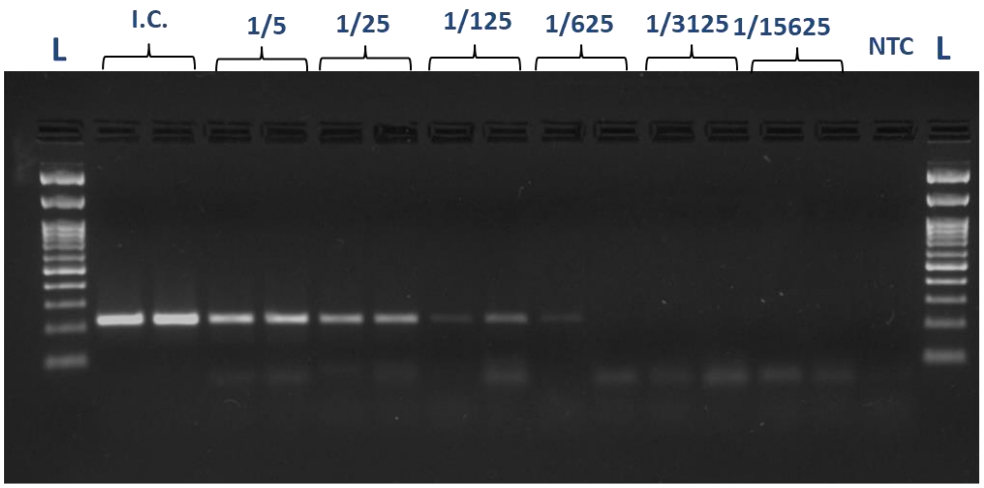


Figure 4.4: Agarose Gel Electrophoresis for the PCR using the B4-B5 primers.
L: 100 bp ladder., I.C.: Initial Concentration: 1.34 ng/µl, NTC: no template control.

The difference in detection sensitivty between the two primer pairs may result from different reasons:

I. Primer design

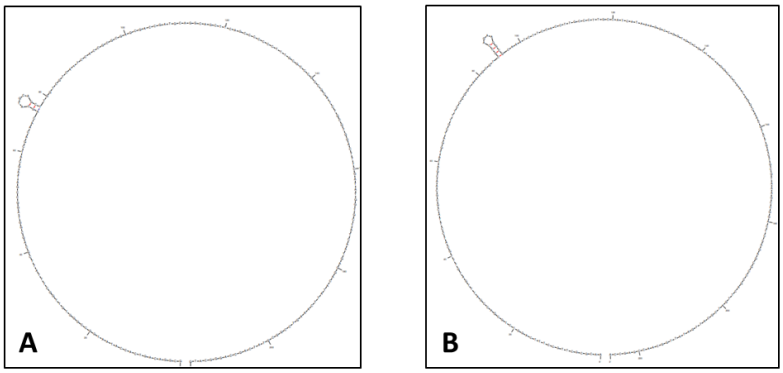
An optimal primer pair for the detection of *Brucella* DNA should have a length between (18-28 bp), G-C content between 50-60% and no strong secondary structures such as hairpins, self-dimers or hetero-dimers (Navarro et al., 2004). These characteristics were checked by IDT (Table 1). The IS1 primers have a G-C content between 50-60%. However, the length of ISF1 is 17 bp and ISR1 is 16 bp which are less than the optimal length. Therefore, the short length of IS1 could be a reason behind the low detection sensitivity of the PCR.

II. Target Copy number

Target copy number play a critical role in determining the sensitivity of any PCR. As the copy number of the target increase, the sensitivity of the test increase (Kalle et al., 2014). The results obtained from Blastn analysis showed that this target exists in an average of ٧ copies per each strain of *Brucella* melitensis. However, the designed primers did not achieve higher sensitivity than B4-B5 primers. According to Riet et al. , the bacterial repetitive sequences are rich with palindromic sequences that tend to form hairpin loops which lead to the dissociation of DNA polymerase from the template (Riet et al., 2017). These hairpin loops may impair DNA amplification and eventually reduce the test sensitivity. Thus, we decided to perform an analysis of the secondary structure of the targeted template sequence.

III. Secondary strucure analysis

To determine the effect of the secondary structure of the target template sequences on the sensitivity, the target sequences were analyzed by mFold webserver at denaturation temperature (95˚C) and at the actual annealing temperatures for each primer set. At the denaturation temperature 95˚C, the results of the IS1 template show that there are no differences between the targeted sequence and B4-B5 template sequence figure 4.5.

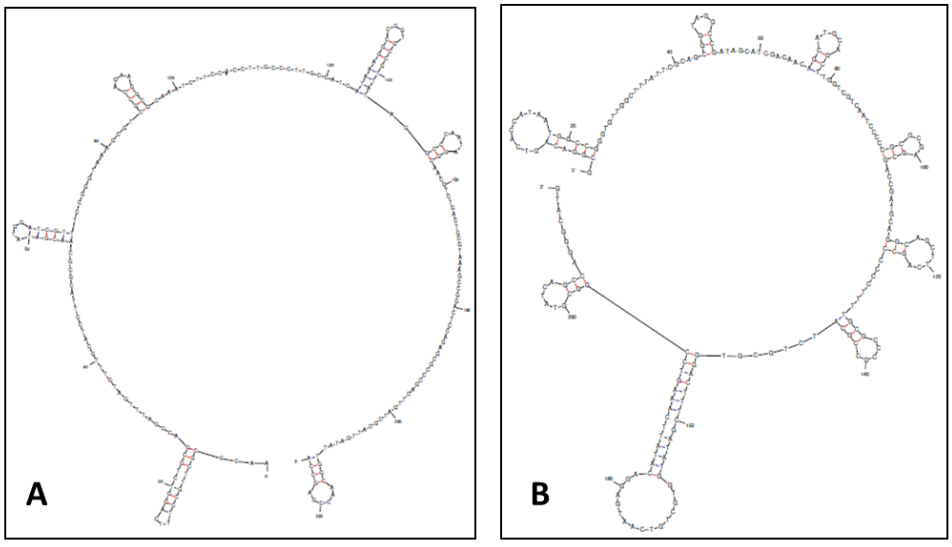


ΔG= 0.52 kcal/mol

ΔG= 1.14 kcal/mol

Figure 4.5: Secondary structure of DNA templates on mfold online software at 95˚C
(A) IS1 template (B) B4-B5 template

However, at the actual annealing temperatures, the IS1 template sequences possess a complex structure because of: 1- a higher number of stem-loop hairpin structures, 2- stem-loop hairpins exhibits much stability and stringency. These strong hairpin structures could cause the polymerase to dissociate from the template which affects the efficiency of the amplification figure 4.6. (Nelms et al., 2011).



ΔG= -6.08 kcal/mol

ΔG= -10.12 kcal/mol

Figure 4.6: Secondary structure of DNA templates for on mfold online software
(A) B4-B5 template (B) IS1 template

Another characteristic of the secondary structure is the value of ΔG. When the value of ΔG decreases, the stability of the secondary structure formation increase. The ΔG for B4-B5 target at exact PCR amplicon size (224 bp) was -6.08 kcal/mol which is more negative than the targets sequences (222 bp) for the IS1 primers (ΔG= -10.12 kcal/mol). These characteristics of the secondary structures could alter the sensitivity of the designed PCR.

4.4 The new IS2 primers design

Because of the low melting temperature of IS1 primers, it is likely that the template hairpin formation may be more stable and thus the template would form the hairpin loop before the primer can bind to the template. To test this hypothesis, we decided to design a new IS primer pair, namely IS2. This new pair of primers has higher Tm and we tried to shift their binding sites to be away from strong hairpin loop complexes. At high Tm, the chance of the primers to anneal to its binding site on the template increases before the formation of the secondary structure.

4.5 Gradient PCR for IS2 primers

In order to determine the behavior of the IS2 primers at different annealing temperatures and to determine the optimal annealing temperature, a gradient PCR was performed using *B. melitensis* Rev-1 vaccine strain DNA at 13.4ng/µl. The annealing temperatures range from 75˚C to 45˚C. As shown in figure 4.7, PCR products relatively faint yet with almost no smear in the range of 75˚C to 69˚C. The yield showed intense bands with relatively low smear around 58˚C to 63˚C. At lower temperatures, some unspecific shorter band start to appear. Therefore, we decided to select 60˚C as an optimal annealing temperature for this PCR to test a serial dilution of *Brucella* DNA to determine the lower limit of detection sensitivity.

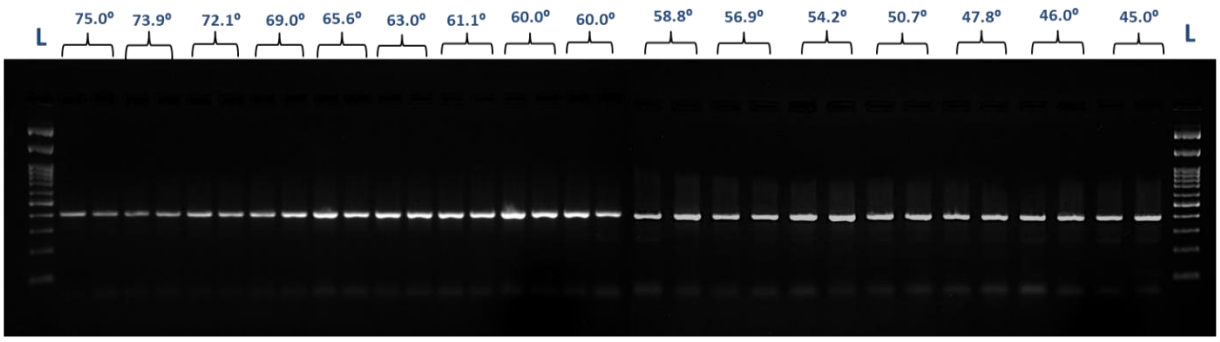


Figure 4.7: Agarose Gel Electrophoresis for the gradient PCR using the IS2 primers
L: 100 bp ladder

4.6 The sensitivity of IS2 primers

The same serial dilution of B. melitensis Rev-1 vaccine strain DNA was tested as a duplicate. As shown in figure 4.8, the IS2 primers could detect the first three concentration (1.34 ng/µl, 0.268 ng/µl, and 0.0536 ng/µl) with good band intensity and the fourth concentration 0.01072 ng/µl gave a detectable yet a faint band. It is obvious that the IS2 set of primers have more detection sensitivity that than the first designed primers IS1. Based on these results, extending the primers length and the increasing in melting temperature have achieved a successful sensitivity that is similar to B4-B5. The results are cosistant with Bounaadja et al., study where the limit of detection was similar between the IS711 and bcsp31 target genes (Bounaadja et al., 2009).

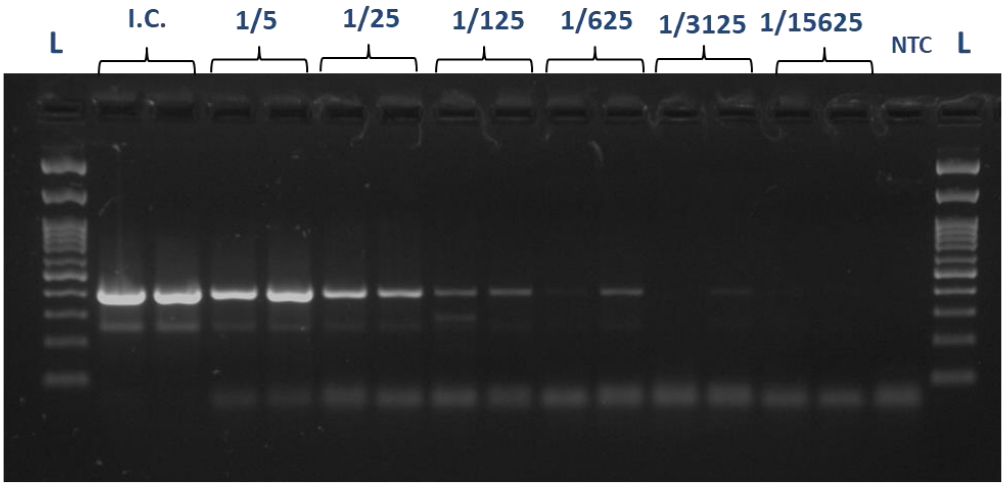


Figure 4.8: Agarose Gel Electrophoresis for the PCR using the IS2 primers
L: 100 bp ladder, I.C.: Initial Concentration: 1.34 ng/µl, NTC: no template control.

4.7 Secondary structure analysis

The template for IS2 primers analyzed in order to compare its structure with IS1 template and B4-B5 template structure. As shown in figure 4.9, the ΔG value fo IS2 template was -23.32 kcal/mol . However, the length of amplicon size is longer than IS1 amplicon. Thus, we divided the ΔG value to the length of amplicon for each primer. The index value for IS1 was - 0.0455 kcal/mol, - 0.0597 kcal/mol for IS2 and - 0.0271 kcal/mol for B4-B5.

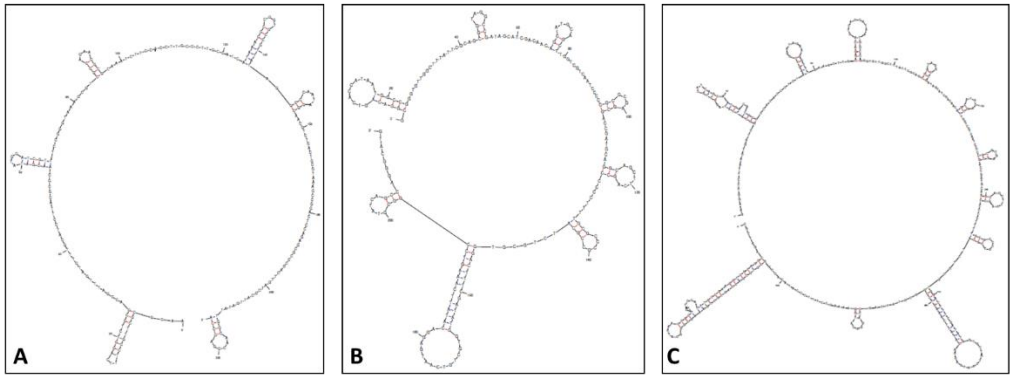


Figure 4.9: Secondary structure of DNA templates for the three PCRs on mfold online software (A) B4-B5 template (B) IS1 template (C) IS2 template

4.8 Seconday structure formation inhibition

The complexity of the repetitive sequences and their capacity to form strong hairpin loops is a common feature of these sequences (Riet et al., 2017). Different solutions were proposed to improve the PCR protocol that targeted repetitive sequences. These solutions focus mainly on testing PCR reaction with varying temperatures of denaturation. Riet et al. study has mentioned the raising of denaturation temperature up to 98˚C would be the hot spot for the amplification of a complex MaSp1 gene sequence (Riet et al., 2017). Therefore, we performed a PCR reaction using IS2 primers at a denaturation temperature of 98˚C to improve the sensitivity of these primers. However, no significant difference in the reaction sensitivity was observed (4.10).

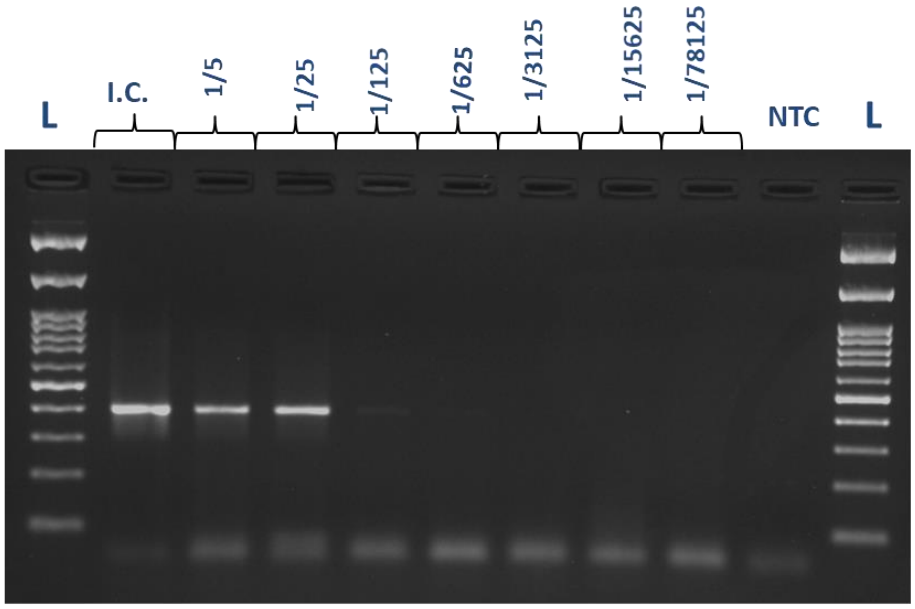


Figure 4.10: Agarose Gel Electrophoresis for the PCR using the IS2 primers at denaturation temperature 98°C

L: 100 bp ladder, I.C.: Initial Concentration: 1.34 ng/µl , NTC: no template control

A variety of PCR additives have been used as a substrate to increase the test's sensitivity and efficiency. One of these additives is dimethyl sulfoxide (DMSO). DMSO inhibits secondary structure formations in the DNA template or the DNA primers allowing the template to stay at the primary structure which facilitate the binding of primers to the template (Frackman et al., 1998). So, we have tried to perform a PCR using the less sensitive IS2 primers with DMSO to reduce the secondary structure formation. The optimal DMSO concentration is usually 5% (v/v%). Two concentrations of DMSO were used 5% and 2.5% in two independent PCR reactions. As shown in figure 4.11, the addition of 2.5% DMSO showed a slight improvement in detection sensitivity when compared with IS1 reaction without DMSO. Frackman proposed that DMSO would alter the annealing temperature (Frackman et al., 1998). So, further analysis of the annealing temperature optimization is needed to enhance the sensitivity.

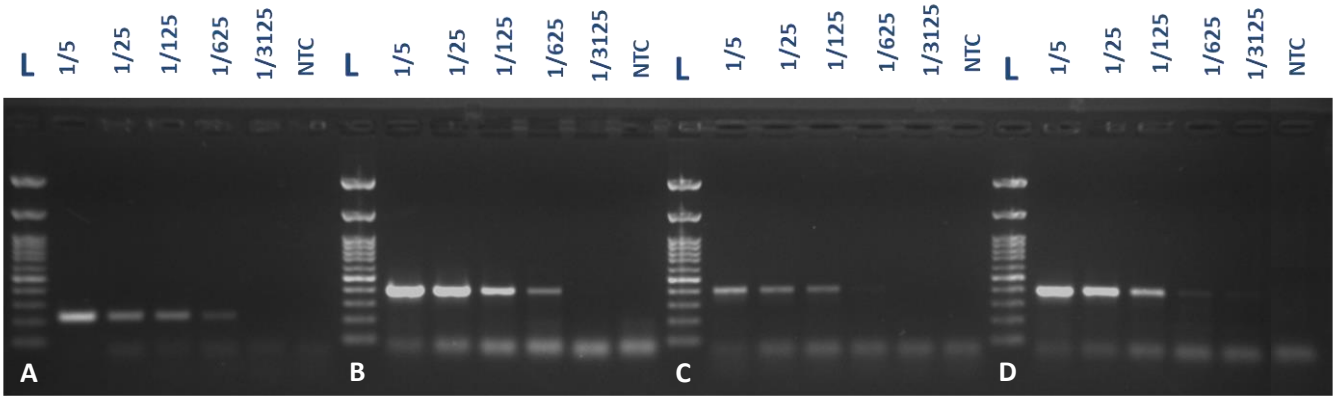


Figure 4.11: Agarose Gel Electrophoresis for the PCR using: From the left:
(A) B4-B5 primers (B) IS2 primers - zero conc. DMSO (C) IS2 primers - 5% DMSO (D) IS2 primers - 2.5% DMSO
L: 100 bp ladder, NTC: no template control.

5. CONCLUSION

We have shown that despite the presence of multiple copies of the IS711 element in *Brucella* genome, two different primer sets (short IS1 and long IS2) that target this repetitive sequence did not exhibit higher sensitivity than B4-B5 primer set that target a gene with a single copy. We proposed that the structure of B4-B5 targeted sequence is flexible enough to sustain linearity at its annealing temperature, while the templates of IS1 and IS2 have strong secondary structures that may impede the stable progress of Taq polymerase as well as stable binding of primers

The longer the primers, the higher the chance of primer-template hybridization. The increase in nucleotide numbers will boost the affinity of primers binding to the template. Besides, higher Tm of the primers means higher chance to anneal to its binding site on the template before the formation of the secondary structure.

As a general recommendation for designing a sensitive PCR, special attention should be paid to the secondary structure formation of the targeted template. We conclude that the secondary structure of the target plays a critical role in affecting the PCR sensitivity. Therefore, it is highly recommended to use *in silico* tools to analyze the secondary structure of the selected target to avoid any strong hairpin that may affect the primer annealing and Taq polymerase amplification efficiency.

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