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Development of loop mediated isothermal DNA amplification (LAMP) test for the detection of active brucellosis infections among diary animals

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Thesis Approval

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Dedication

I dedicate this work to my Parents

I dedicate this work to my sisters and my brothers,

I dedicate my work to all teachers who teach me,

I dedicate my work to my friends

Ahmad Musallam Makhamreh

Declaration

I declare that this thesis for the master's degree in biochemistry and molecular biology has been done as a result of my own research except others, cited here will not be submitted to another university or any other institution.

Signature: 

Ahmad Musallam Ibrahim Makhamreh.

Date: 26/04/2021

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Abstract

Brucellosis is a zoonotic, infection caused by bacteria from the genus *Brucella*. It is a socioeconomic threat that affects various mammalian species and it can also be transmitted to humans. However, because of the non-specific nature and shared symptoms in other febrile illnesses as well as its slow growth on the blood culture, diagnosing brucellosis is a very difficult task.

The Rose Bengal test is an internationally accepted screening for brucellosis. These are not always very specific as the antibodies can cross-react with other gram-negative bacteria, these tests have limited value in the detection of infection at early stages and may give a false negative result for a short period after infection; they too lack sufficient sensitivity, especially during long chronic cases or high specificity endemic areas.

A LAMP assay was developed by us to diagnose the brucellosis. It is a highly sensitive, simple, fast cost-effective method that requires 60 minutes to complete the reaction at the temperature of 63 °C. The strand displacement reaction occurs under isothermal conditions and includes four distinct primers, each of which targets six different areas in the target gene. The cross-reactivity with the other bacteria was very absent. It is more effective than the classic PCR (cheaper, easier, faster and requiring no equipment while achieving the same results).

A blood analysis was done on the 5000 sheep. Of these 200 (4%) were just positive. From the positive results, we re-examined using the LAMP method that has been developed by ourselves (200 samples). However, with the creation of the LAMP method we could eliminate the interaction between other microorganisms and get rid of the incorrect positive result caused by antibodies against *Brucella* in blood after a previous infection. , The LAMP method differs from the serology approach in that it relies on bacterial DNA detection as opposed to the antibodies directed at bacteria; It also eliminates all of these difficulties along with others caused by using the serologic approaches.

ملخص

تطوير تقنية ال LAMP للكشف عن بكتيريا البروسيلا النشطة في الحيوانات المنتجة للحليب

الحمى المالطية: هي عبارة عن مرض ناتج عن الإصابة ببكتيريا تسمى البروسيلا، تصيب الكثير من الثديات وخاصة الأغنام والأبقار. كما أنها تنتقل إلى الإنسان عن طريق العدوى من تلك الحيوانات، تؤدي إلى حدوث اجهاض متكرر وضعف عام عند الحيوانات التي تصيبها، كما أنها تسبب صداعا حادا وارتفاعا بدرجات الحرارة خاصة في الليل وضعفا عاما عند إصابة الإنسان.

الطريقة الشائعة للكشف عن بكتيريا البروسيلا هي الطرق السيرولوجية مثل فحصRose-Bengal او فحص ال ELISA، حيث إن هذه الطرق تعتمد على الكشف عن وجود الأجسام المضادة لهذه البكتيريا، وأن الأجسام المضادة للبكتيريا تبقى مدة طويلة داخل الجسم بعد الشفاء من الإصابة، ووجود الأجسام المضادة للبروسيلا داخل الجسم لا يعني أن الإصابة حالية. كما أن نتيجة هذا الفحص تتداخل مع الكثير من أنواع البكتيريا الأخرى المشابهة للبروسيلا.

لقد قمنا بتطوير طريقة ذات دقة عالية وبفعالية تفوق جميع الطرق المعتمدة للكشف عن البروسيلا ذات مزايا ممتازة، و خطوات بسيطة سهلة ، بالإضافة إلى التكلفة المادية القليلة نسبيا وعدم حاجتها لأدوات معقدة، تدعى هذه الطريقة (Loop Mediated Isothermal Amplification) . إنها تعتمد في كشفها عن البكتيريا على ال DNA لهذه البكتيريا وهذا ما يمنحها الدقة العالية ، وعدم تداخل نتيجة هذا الفحص مع الأنواع الأخرى من البكتيريا المشابهة للبروسيلا. وتحتاج إلى أربعة من Primer تربط على ستة مواقع مختلفة على سلسلة ال DNA , لا تحتاج إلى درجات حرارة مختلفة كتلك المستخدمة في تقنية ال PCR، حيث إن الكثير من الميزات لتقنية ال LAMP تمنحها التفوق أيضا على تقنية ال PCR التي تحتاج إلى أجهزة ثمينة معقدة ووقت طويل.

قمنا بعمل هذه الدراسة باستخدام 5000 عينة مأخوذة من الأغنام والماعز، كما ان هناك عدد قليل من عينات الابقار والجمال ضمن هذه العينات من مناطق مختلفة من فلسطين، حيث تم فحص تلك العينات بشكل أولي من خلال فحص Rose-Bengal, كانت عدد النتائج الإيجابية للبروسيلا تساوي 200 عينة (كانت نسبة الإصابة تعادل4%). حيث قمنا بإعادة فحص العينات الإيجابية باستخدام تقنية ال LAMP التي قمنا بتطويرها بالشكل الذي يتناسب مع ال DNA للبروسيلا واستبعاد جميع أنواع البكتيريا الأخرى التي من الممكن ان تتداخل مع البروسيلا. بينت النتائج التي حصلنا عليها الدقة العالية لفحص LAMP مقارنة مع فحص الروز بنجال بالاعتماد على تقنية ال PCR في مقارنة النتائج. حيث انها تماثل تقنية ال PCR من ناحية دقة النتائج وتتفوق عليها من ناحية سهولة الاستخدام ,اقل تكلفة وعدم حاجتها الى أجهزة.

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List of abbreviations:

LAMP	Loop Mediated Isothermal Amplification
UTI	Urinary Tract Infection .
POC	Point Of Care .
IgM	Immunoglobulin M
IgG	Immunoglobulin G
IgA	Immunoglobulin A
RBPT	Rose Bengal Plate Test
ELISA	Enzyme Linked Immunosorbent Assay
LPS	Lipopolysaccharides
CFT	Complement Fixation Test
RBT	Rose Bengal Test
PCR	Polymerase Chain Reaction
AMOS	Abortus-Melitensis-Ovis-Suis
FIP	Forward Inner Primer
F3	Forward outer primer
BIP	Back ward Inner Primer
B3	Back ward outer primer
Tm	Temperature

Chapter 1:

1. Introduction and review of literature

Brucellosis is a zoonotic disease due to the bacterial genus *Brucella* that are short non-motile, non-sporing intracellular gram-negative coccobacilli or rods that grow slowly on ordinary media. While *Brucella* is aerobic, some of the strains require an additional supply of carbon dioxide. All the *Brucella* strains are catalase and superoxide dismutase-positive although most of them are also oxidase-positive. A majority of the species, especially *B. ovis* and *B. suis*, will test positive for nitrate reduction while reducing it into nitrite. The physicist J. A Marston first identified brucellosis in 1859 through the British Royal Army Medical Corps and he connected it with some troops of Malta during the Crimean War (Vassallo, 1992). Furthermore, a different physician (David Bruce) succeeded in identifying the bacteria from the spleen of an infected man in Malta and was subsequently named after him. Brucellosis is among the most frequent zoonotic diseases and globally about 500,00 cases are reported annually (Atluri et al., 2011).

Brucellosis is one of the most widespread zoonotic bacteria worldwide, and it has a high prevalence in the Mediterranean region, the Arabian Peninsula, the Indian subcontinent; Mexico as well as in South and Central America. Human brucellosis has been diagnosed in the US historically, and this trend was diminished by cattle immunization programs, as well as test-and-slaughter methods to eliminate bovine brucellosis. In the United States, there are in fact less than 0.5 cases per every hundred thousand individuals (Pappas et al., 2006), with most of these being centred on border areas between Mexico and the US. A leading cause of human suffering and monetary losses.

1.1 History:

In his turn, a British army surgeon David Bruce (1855-1931) isolated from the spleen of a Brucellosis patient who had died in 1886 a cocco-bacillus that he named Micrococcus melitensis (Bruce, 1887). In Malta, the average annual incidence in 1901–6, with a mortality rate of 10.4 percent and 2.3 percent respectively, of 652 civilian and 605 military cases (Eyre, 1908). The people who take goat milk and have direct contact with the animals have been connected to human diseases. It was an endemic condition, but it was mistaken for other diseases, particularly malaria.

A related microbe was isolated from cattle in 1897 and also from pigs in 1914. About 1920, the genus was renamed *Brucella* and its species were also designated as *Br. melitensis*, *Br. abortus*, *and Br. suis*. The disorder was named Brucellosis, which has many names with the prevailing undulating fever in the U.S. until the 1940s. The American outbreaks of fever were first made known in 1903 by exposure, possibly via the infective agents from the Spanish-American war (Craig, 1903). In 1924, Baltimore produced the human blood culture Br. abortion (rather than *Br. melitensis*) (Keefer, 1924).

However, therapeutic vaccines were more noticed in the 1930s (Dalrymple-Champneys, 1935) but their impact "seemed rather to be obscure. But many drugs were tested. Particular interest seems to have been reflected in metallic medicines available to cure other diseases: For instance, I.V. Neosphenamine, the IV Mercurial Mercurochrome (Mercurial) (Neumann 1936), I .M Bismuth-mercury Enesol, also IM Antimonial (Fouadin) (Richardson, 1938) However, sulfanilamide was the first drug that was extensively studied and which seems to have offered a rapid result (Blumgart, 1938; Bynum, 1939). The first of several studies in English appeared in 1938. but there were failures even in 1939. After sulfonamides, monotherapy also failed.

Between 1947 and 1951, the introduction of antibiotic therapy was not fully trusted either. Streptomycin with intramuscular administration was the earliest hope (Pulaski and Amspacher,

1947), although another study considered monotherapy ineffective. The latter research was the first to discover the possible efficacy of streptomycin and also oral sulfadiazine (Eisele and Mccullough, 1947). This was demonstrated very easily (Spink et al., 1948). However, the method was never very feasible in the case of parenteral administration for streptomycin. The first chlor-tetracycline test (Aureomyc) was initially linked with sulfadiazines, performed subsequently in Mexico in 1948 but only alone. This medication possesses the functional advantage over oral streptomycin and does not cause any ototoxicity. In the same year, drugs to be mentioned briefly in this brief review include Chloramphenicol (Chloromycetin) and also Oxy-tetracycline (Terramycin) (Harris, 1950).

1.2 Taxonomy:

Brucella taxonomy and nomenclature have been a great bone of contention for what is considered impossible to reconcile genetic variation with such wide phenotypic differences that are used in the classification into species and subspecies under the genus *Brucella spp* (Whatmore 2009). It has been known since the late 19th and also early 20th century. They have been characterized primarily in terms of the host species from which they were obtained and are responsible for morbidities that cause a marked disease burden still today.

The disease-causing agent of the *Br. melitensis* has been confirmed in the British Army. Staff were established in Malta by David Bruce (Bruce, 1887) But the thanks go to Themistocles Zammit who demonstrated that human infection was a result of the goats' milk (Wyatt, 2005). Similarly, over the following decades, *Brucella* species has been shown to be associated with other hosts, such as *Br. abortus* in cattle (Bang, 1906) in sheep, (Buddle, 1956), *Br. canis* in dogs

(Carmichael and Bruner, 1968). *Br. neotomae* in desert wood rat (Stoenner and Lackman, 1957) and *Br. suis* in swine (Huddleson and Hallman, 1929).

Although each of these has been categorized summarily as a class 3 biohazard, when compared to the particular host for example humans there are many distinct differences in the disease they cause from differing pathogens. *Br. melitensis* is one of the most virulent forms and it spreads very quickly. Also, *Br. abortus* is no less dangerous. *Br. suis* has a moderate severity. While *Br. Canis* features a moderate level of risk, *Br. ovis* is almost innocuous. This can be attributed to the relative rarity of human and animal diseases as well as to differences in virulence that occur from time to time. To validate the identity of the species as well as monitor their subdivision for effective detection and epidemiological surveillance, a phenotypic testing method was created in 20 th century (Alton et al., 1988).

Table 1: Host preference for domestic animals by *Brucella* species

Species	**Natural host**	**Zoonotic potential**	**Clinical signs**	**Transmission**	
Brucella melitensis	Small ruminants	High	Female: abortion, weak offspring, reduced milk yield Male: infertility, orchitis, epididymitis (rare)	Oral: ingestion of contaminated placenta, aborted fetus, contaminated milk	(N Xavier et al., 2010)

Brucella abortus	Cattle	Moderate	Female: abortion, weak offspring, reduced milk yield Male: infertility, orchitis, epididymitis (rare)	Oral: ingestion of contaminated placenta, aborted fetus, contaminated milk	(N Xavier et al.,2010; Xavier et al., 2009)
Brucella suis	Pig	Moderate	Female: abortion, weak offspring Male: infertility, orchitis, epididymitis, osteoarticular disorders	Oral: ingestion of contaminated placenta, aborted fetus, contaminated milk Venereal: breeding using contaminated semen	(Ewalt et al., 1997)
Brucella canis	Dog	Mild	Female: abortion at 45–55 days Male: infertility, orchitis, epididymitis Both genders: bacteremia	Oral: ingestion of contaminated placenta, aborted fetus, contaminated milk Venereal: breeding using	(Lucero et al., 2010)

				contaminated semen	
Brucella ovis	Sheep	Absent	Female: abortion, weak offspring (rare) Male: infertility, orchitis, epididymitis	Oral: close contact between rams Venereal: use of infected rams during mating season	(Júnior et al., 2012)

1.3 Transmission:

There are many ways to cause the transmission of *Brucella spp*: Person-to-person transmission, contamination from a polluted area, and environmental exposure usually occur because of close contact with infected animals and the food is transmitted.

1.3.1 Person-to-person transmission:

This is exceedingly rare. There are some occasional instances where the circumstantial evidence suggests that the path of transmission was very close to personal or sexual contact. What is more possible to be of significance as a transmission mode by blood donation or tissue transplantation (Yumuk and O'Callaghan, 2012). Actually, bone marrow transplant causes a very serious problem. The transmission of relatives close to patients with brucellosis is almost

impossible; however, some fundamental measures should be observed. Lab workers pose a significantly greater risk of stealing blood from patients.

1.3.2 Infection from a contaminated environment:

Brucella spp may remain in the dust for an extended period. Dung, water, slurry Fetuses; soil meat and dairy products have been aborted. Various features, including the substrate composition, diversity of species, temperature and pH requirements for light and other microbial contaminants depend on how much time lives (Sammartino et al., 2006).

Animals that move through populated areas or are kept in close quarters to buildings, particularly if abortions occur, tend to create serious contamination of roadsides yards and marketplaces. Touch contamination also arises from the soiled surfaces that may cause dirt and other polluted soil to dry dung, etc. The inhalation of diseases of Brucellosis will then develop exposing individuals. Livestock that were recently aborted or rainwater runoff from contaminated fields can also pollute dams and other aquatic bodies (Van Bressem et al., 2009).

1.3.3 Occupational exposure:

The risk of brucellosis infection in some occupations is very high. This includes persons who deal with farm animals, especially cattle, sheep, goats and pigs: farmers, farm workers, animal caretakers' stock-keepers shepherds goatherds pig keepers veterinarians and inseminators are influenced by close contact with animals experiencing disease or through exposure to highly contaminated sites. Methods of infection include inhalation, conjunctival contamination, ingestion through accidental injuries such as cuts or abrasions and also aerosols and skin contact.

The families of the farmers and animal breeders may also be at risk, given that animals need to live near living housing; domestic exposure often overlaps or is inseparable from occupational exposure. In some places, animals may stay in the yards of the houses and even be taken inside during severe weather. This has caused many homes to become contaminated in the event of a recent abortion. Other modes through which *Brucella spp* may be introduced in the home are by using dried dung as a heat. It must be noted that brucellosis is often clustered as a case when it occurs in the family or tribal set-up primarily linked to one contaminated food source, and oftentimes follows an epidemic of animals.

People who are engaged in the processing of animal products may be exposed to many severe risk factors from brucellosis. These people include slaughtermen, butchers, meat packers, fetal calf serum collectors' skin and wool processors on dairy workers. These may occur via ingestion, absorption, mucous contact and also skin contact or penetration; hazards include direct contamination as well as that is environmental. We often disregard the workers employed in managing field premises, factories or plants used to produce animal products as classes exposed to occupation yet these people are at a high risk of environmental pollution (Corbel 2006).

1.3.4 Food-borne transmission:

This is usually the main factor leading to Brucellosis in urban centers. For most of the population, the major means by which they contracted infections were through unheated milk consumption or other products prepared from it. Milk that is contaminated for the cows, horses, goats or camels. *Br. melitensis* is especially dangerous as it contains a colossal number of bacteria that are consumed in large quantities. Butter, cream or ice cream from such milk also pose a high risk. In the Mediterranean and Middle East, soft cheeses produced from sheep or goats' milk with

rennet are considered one of the widespread sources of diseases. In reality, *Brucella* will be unconcentrated during the cheese-making process; however, in this form of product the species can persist for several months. In the cool weather, these cheeses should be processed at least six months before use.

The risk associated with these cheeses is much lower as they are prepared by lactic and also propionic fermentation. Similarly, yoghurt and also sour milk are less toxic. While *Brucella* quickly perishes as the acidity drops below pH 4, and very rapidly at below pH 3.5; unless good hygienic practice is followed, equipment used in the transport or processing of infected milk or other raw materials may not lead to contamination of non-infected foods but Meat products are associated with infection less often primarily because they are normally But it is only a habit of but Muscle tissue usually contains relatively low levels of *Brucella* organisms; however, significantly higher concentrations are generally present in the liver, kidney, spleen udder and also testis. In some countries, raw or undercooked dishes made from these organs can be eaten. Fresh blood may be drinking, either by itself or in conjunction with fresh milk creating a clear and imminent threat.

Brucella spp. urease activity might help in the persistence throughout the stomach. Two urease operons were identified in the *Brucella spp* chromosome1 (Sangari et al., 2007). Genome during the sequencing process. *Br. ovis,* which is transmitted sexually rather than orally, is the only *Brucella spp* that does not generate urease. The lack of the enzyme is most likely to blame for this incapacity (Fekete et al., 2019).

1.4 Epidemiology:

While brucellosis is considered a zoonotic disease, the infected animals act as vectors for the transmission. The primary species are the main animals that produce food: cows, horses, pigs and swine. Other species are less significant but they are locally very common as bison, camels, horses, goats, reindeer and also yaks. Pollution sources in certain countries. Recent diseases have also been reported in aquatic mammals such as dolphins, porpoises and seals; therefore, they are an emerging hazard of work for the individuals who handle infectious tissues from them (Van Bressem et al., 2009).

Among others, the nature of *Brucella* to which an individual is exposed mainly determines the risk of infections and their severity. This will depend on the type of host animal that is an infection reservoir.

Brucellosis is seen around the world, particularly in developing nations; higher levels are noted in where Arabia peninsula and the Mediterranean area indian subcontinent Mexico South and Central America *Brucella* species occur at variable frequencies throughout the United States animal immunization programs test-and-slaughter methods have reduced human cases. American cases of whooping cough are estimated to be fewer than 0.5 per every one hundred thousand US citizens, and this disease is highest prevalent in the border region between Mexican Americans (Pappas et al.,2006).

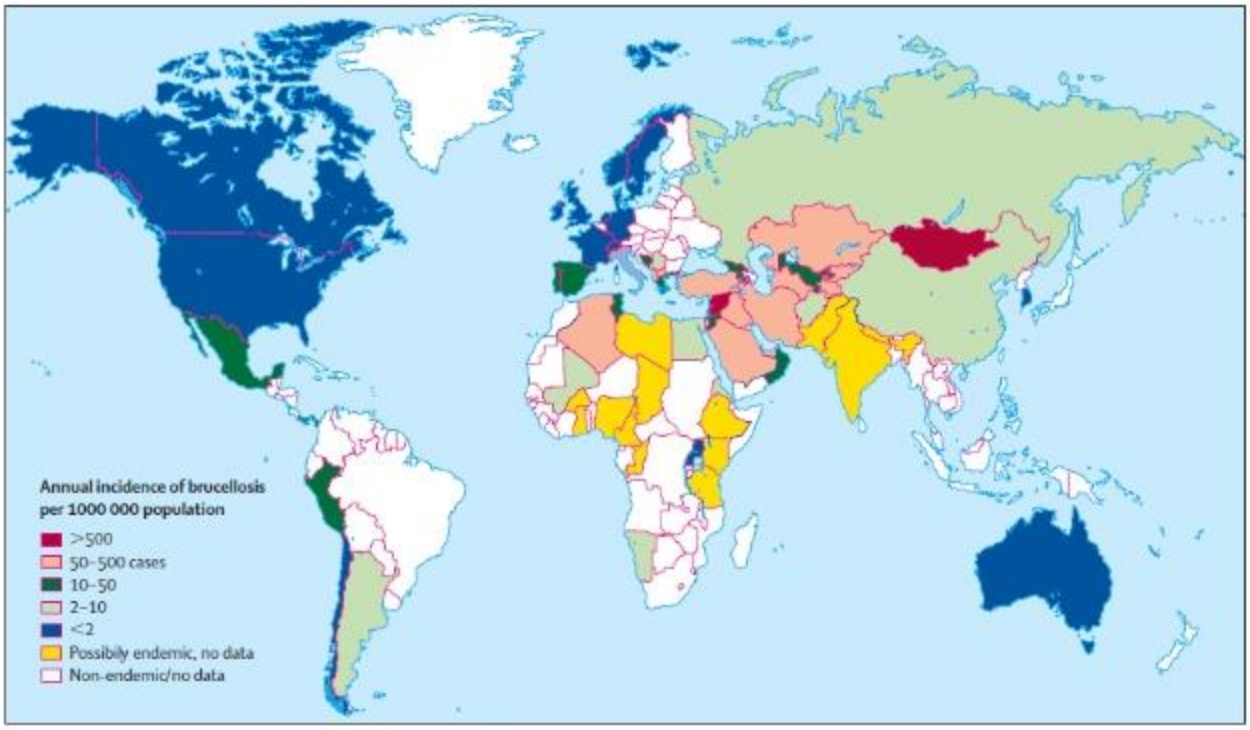


Figure 1: Show the Worldwide incidence of human brucellosis (Pappas et al., 2006).

Among the historically believed endemic areas, France and most of Latin America have managed to contain the infection. However, on the other hand, new human brucellosis foci have appeared mostly in (Central Asia), and the situation is changing quickly in several near-eastern countries or areas such as Syria. In addition, the disease is still present in Europe and America with varying degrees of intensity (Pappas et al., 2006).

1.4.1 Brucellosis in Palestine:

Brucellosis considered being one of the major zoonotic diseases in Palestine. The first case was documented in the Hebron region in 1973. In 1998 Hebron region was reported to have the highest incidence rate in Palestine (139.9/100,000), followed by Jericho and Bethlehem (Husseini and Ramlawi, 2004) Many cases of human brucellosis in Palestine are caused by ingestion from unpasteurized dairy products. An important source of infection was also

occupational exposure to diseased farm animals. In Palestine, the incidence of brucellosis has increased steadily since 2012, as in recent years the incidence of this disease has increased dramatically according to statistics, the areas of the southern West Bank, especially the Hebron governorate, showed very high rates of infection compared to the rest of the Palestinian areas. According to statistics carried out by the Palestinian Ministry of Health in 2011, the total number of human cases was 179, of which 125 were in the Hebron Governorate, 31 in the Bethlehem Governorate, and 23 in the rest of the Palestine governorates. An increase in disease numbers were reported in year 2018 in the areas of southern Hebron and Bir al-Sabe, due to the uncontrolled trade of sheep between the areas of Bir al-Sabe and southern Hebron. As the incidence of Maltese fever in the Bir al-Sabe regions is much higher than in the south of Hebron.

The reports on human brucellosis from the Palestinian Ministry of Health were analysed from 2000 to 2020. The total number of cases recorded over this time period was 7935, with an average annual incidence rate of 9.4 cases per 100,000 people. Hebron With a total of 6106 cases (77 %) and an annual average incidence rate (AAIR) of 45.6 cases per 100 000, it was the most endemic governorate. In Hebron, the average number of patients per year was 290.8 As shown in Table 2 (Amro et al., 2021).

Table 2: Distribution of human brucellosis cases in the West Bank from 2000 to 2020

Governorate	Number of patient	%	per 100 000 population	AAIR
Hebron	6106	77.0%	45.6	290.8
Bethlehem	697	8.8%	16.6	33.2
Ramallah	274	3.5%	4.3	13.0
Jerusalem	169	2.1%	2.4	8.5
Nablus	158	2.0%	2.2	7.5
Jenin	124	1.6%	2.2	5.9
Salfit	117	1.5%	9.2	5.6
Qalqilya	105	1.3%	4.9	5.0
Jericho	81	1.0%	10.3	3.9
Tulkarem	68	0.9%	2.0	3.2
Tubas	36	0.5%	2.8	3.0
Total	7935	100%	9.4	377.9

1.5 Clinical Features

Brucellosis is an often insidious disease with nonspecific early symptoms, regardless of the species infected. In humans, the incubation period for brucellosis is usually 2 to 3 weeks, but it can sometimes extend many months. Acute infection could go unnoticed and lead to chronic illness, with symptoms disappearing years later. The most prevalent symptoms are cyclical (undulant) fever, nocturnal sweats, and neuropsychiatric symptoms including headache.

Malaise, sleeplessness, and arthralgias are also common symptoms. Specific clinical signs are less prevalent than systemic signs: arthritis, organ involvement and genitourinary signs develop, generally in that order of frequency. Pregnant women may experience spontaneous abortions (Khan et al., 2001).

Endocarditis, the most severe complication and the most usually linked with *Br. melitensis* infection, is uncommon (2% of cases), but accounts for the majority (80%) of deaths (Peery and Belter, 1960). Clinical symptoms can differ based on the *Brucella spp* that is causing the illness. In a recent study of brucellosis patients in the United States, *Br. melitensis* infection was found to be more likely to induce acute, systemic disease than other *Brucella spp*. Infections (Troy et al., 2005)

1.6 Diagnosis and Identification:

There are two types of diagnostic tests: The latter include those that demonstrate the presence of an organism and also those that are used to detect immune responses on their antigens. Isolation of *Brucella* is conclusive proof that the animal is infected. However, cell-mediated immunity is required to destroy and eliminate the *Brucella spp*. Although their role in infection prevention is very limited, antibodies are also essential for diagnostic purposes. IgM level increases in the first week of the infection and subsequently, after 14 days IgG levels become elevated. Elevated or growing titer of the antibody can really help to diagnose. IgG and IgM levels fall after the treatment, IgG decreases faster and IgM can persist for months to years at low titers , but an elevation of these measures is typically for chronic infection or reappearance at 6 months where high titers of both lie. Serology should be read with caution because the

negative test results cannot exclude a recent infection and after recovery antibodies (especially IgG) remain (Ariza et al., 1992).

Brucellosis can also be diagnosed by culturing the bacteria in the blood, bone marrow or tissue cultures under high biosafety to keep the laboratory team free from infection. Since *Brucella* has a slow growth rate in vitro, it is crucial to watch over its development for up to 28 days. Some new blood culture systems, which are constantly monitored can detect growth in 7 to 10 days. Reaction-based screening of the polymerase chain, on its part, can contribute to a much faster diagnosis and detect the bacteria within 10 days after inoculation.

1.6.1 Serological methods

The most useful diagnostic approach is still the determination of a specific antibody in serum or milk. Screening all the samples with a cheaper and rapid test that is specific enough to detect multiple infected animals in most cases will prove to be the most efficient and cost-effective method. Samples that are positive for screening then use the confirmatory tests, more complicated and specific ones to determine the final diagnosis (Stemshorn et al., 1985).

However, only the studies conducted internationally and used antigens standardized with International Anti-B are entirely necessary. Serum *Br. abortus* is included. Appropriate quality management sera must accompany each series of samples, and the tests should be performed in replicates if the criteria for control are not met. The results of serological analyses should be compared against the incidence rates for infections, the vaccine-use situation and false positive rates because other species are involved. In the case of any other laboratory-based testing, a good "audit trail" must be established to enable individual animal identification, quantification and test report so that there is clear confidence in the subject relationship with the outcome.

1.6.1.1 Rose Bengal Plate Test (RBPT):

The RBPT is an agglutination technique also known as the *Brucella* antigen test or buffered test (Stemshorn et al., 1985), which uses a suspension of smooth *Br. abortus* cells colored with the buffered Rose Bengal dye at 3.65 pH. The measurement of IgM, IgG1 and IgG2 at the neutral pH is enabled by this test. As the most used method in Palestine, this test is a diagnostic tool with many false positive results that may be due to the reaction of IgM against previous vaccination animals on the significant part. The availability of this test will allow the recording history of animal contact with *Brucella* species under conditions in which vaccination is not routinely carried out. It is an internationally recommended test for brucellosis screening in small ruminants, but the lack of antigen standardization is also a problem. The acidity of the antigen elevates the specificity while adjustments to the sample's temperature or that in which a reaction takes place may affect sensitivity and/or specificity (Alton, 1981).

The RBPT is a very simple test in which drops of stained antigen and serum are placed together on the sheet with any signs of agglutination taken to be indicative positive reaction. The test is a very useful screening procedure, but in animals on an individual basis, especially vaccinated ones this can be too sensitive to diagnose. However, the process can be automated; however, this also involves special equipment.

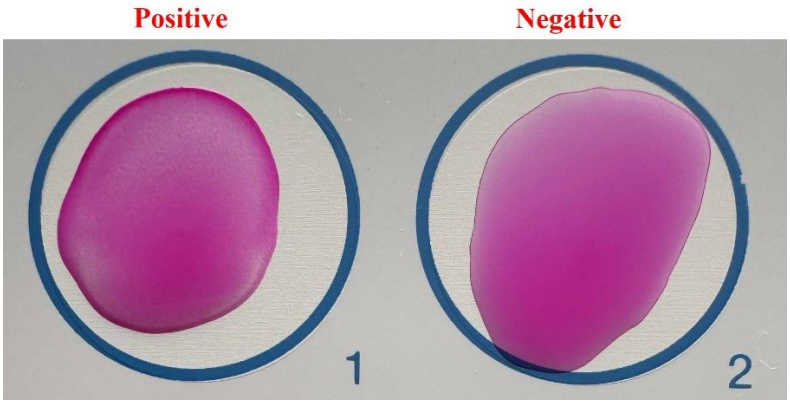


Figure 2: Rose Bengal plate test.

1.6.1.2 Enzyme linked immunosorbent assay (ELISA):

ELISA has become popular serologically as a standard test for the diagnosis of brucellosis, this measures antibodies IgG, IgA and IgM and this allows a better interpretation of the medical situation. The diagnosis of brucellosis rests on the antibody detection to smooth lipopolysaccharide. The detection of IgG antibodies is way more sensitive compared to the identification using IgM as an indicator for cases of brucellosis (Agasthya et al., 2012; Araj, 2010). In turn, the combination of ELISA IgM and also IgG tests is recommended for case identification as well as an accurate diagnosis in suspected cases since it has proved to be one of the most efficient methods used during brucellosis detection and diagnostics.

Because they are relatively stable, simple to perform with very little equipment and also available commercially in kits as a ready supply of raw materials for the test, ELISA tests display superior sensitivity and specificity. They fit well in smaller labs than CFT and, the ELISA technology is currently being used for diagnosing various diseases among animals as well as humans. Although ELISAs may in general be used for serum analysis of both animal and human organisms, the results can vary from one laboratory to another due to the differences in actual methods. However, not all the issues of standardization have been completely resolved. A single dilution test is typically conducted for the screening. It is important to note that although ELISA are more sensitive than the RBT, they frequently do not detect animals positive for the RBT. Furthermore, one should bear in mind that the specificity of ELISA is about two times more than that of RBT or CFT.

1.6.1.3 Complement Fixation Test (CFT)

It is an immunological diagnostic test that depending on whether complement fixation occurs, can detect either a specific antibody to be present in the serum or a specific antigen. It is

widely used for the diagnosis of infections, especially those that are not easily cultured from the microbes.

Although CFT has a relatively good performance, requiring well-equipped laboratories and expert technicians, this method is very challenging to perform. It will be very rewarding if these are availed and the research is carried out at regular intervals while taking quality assurance more seriously. The sensitivity and specificity of the CFT is acceptable, but needing good laboratory facilities and skilled personnel, it is a challenging technique to conduct. It will be very satisfactory if these are present and the research is conducted frequently with good regard to quality assurance. Due to the incidence of the prozone phenomenon, it is important to titrate each serum sample where low dilutions of certain sera from infected animals do not repair the complement. This is because of the character of high numbers or levels for non-complement fixing antibodies with different kinds fighting each other to bond on the antigens. In higher dilutions, these disappear and the complementation is achieved. However, if they are tested using only one dilution, some positive samples would not be detected. Otherwise, if there is no antigen and in the case of a contaminant bacterium or any other factors present in the serum samples that repair complements by inducing positive results during this test. Such “anti-complementary” responses make the test very pointless and no CFT result is possible to obtain (Adone & Ciuchini, 1999).

1.6.2 DNA based test:

1.6.2.1 The polymerase chain reaction (PCR)

The PCR is a sensitive and quick technique that makes it possible to diagnose brucellosis quickly and accurately without the limitations of traditional methodology (Gupte and

Kaur, 2015), Several genus-specific PCR systems have been developed using primary pairs targeting 16SRNA sequences and genes of different external membrane proteins (Bricker, 2002), The PCR is based on the polymorphism of the insertion sequence IS711 in the chromosome of *Brucella*.

The first PCR-based test for brucellosis was implemented in 1990 (Fekete et al., 1990). The first species-specific multiplex PCR, used to classify and distinguish was called the Abortus-Melitensis-Ovis-Suis (AMOS-PCR) assay. The PCR is based on the polymorphism of the insertion sequence IS711 in the *Brucella* chromosome resulting from species-specific localization (Bricker and Halling, 1994).

1.6.2.2 Loop-mediated Isothermal Amplification (LAMP)

Another molecular tool used is the LAMP test whose many advantages include its ease of use, rapidity and convenient detection. A simple and, moreover, cheap system such as a water bath or heat block that maintains a constant temperature of 63°C is very suitable for conducting this experiment (Ohtsuki et al., 2008). In contrast to PCR, the reactivity is detected directly with the naked eye without any electrophoretic analysis. This is because it enjoys many advantages such as simplicity, and a low cost per sample (the cost for each one ranging from 3 to 6 times cheaper compared with PCR and real-time PCR) than other molecular methods requiring expensive equipment.

These techniques are very sensitive and it enables the detection of the target DNA that is at low levels. Positive results, however, provide no rational data on the degree of infection and do not indicate whether replication or cause of disease in a species is investigated by the pathogen. The carrier status and the pathogen viability cannot be established by using DNA probes.

LAMP is an in-house, nucleic acid amplification method developed by Eiken Chemical Co., Ltd. It is simple, rapid specific and less costly (Notomi et al., 2000). It has four primers that recognize the six different locations on the target gene, and the displacement reaction is conducted at a stable temperature. The mixture of primers, DNA polymerase with the strand displacement activity and substrates can be incubated at a constant temperature (about 65°C) forming amplification in one step. The amplification copies the DNA up to 109-1010 orders of magnitude in just 15 –60 minutes, providing for a very high yield efficiency. If an amplified product is found, this could be indicative of the presence of the target gene because it has a high degree of specificity (Notomi et al., 2000).

The LAMP technique is a gene amplification technique that includes the following features:

1. There is only one enzyme needed, and the amplification reaction takes place in an isothermal environment.
2. A high level of specificity is achieved by using four primers that recognize six distinct regions on the target.
3. High amplification efficiency makes for rapid amplification.
4. It generates a large number of amplified materials, allowing for easy identification.
5. A step to denature double stranded DNA into single stranded DNA is needless (Notomi et al., 2000).

Table 3: Comparison between PCR and LAMP:

LAMP	PCR
Method of amplifying DNA using a uniform temperature and specialized DNA polymerase.	Method of amplifying DNA, using change in temperature to separate and anneal the primers.
Require 4 different primer	Requires one forward and one reverse primer.
Isothermal reaction, one temperature 60-65 C.	Cyclic reaction variable temperature
Detection limit is greater.	Detection limit is lower.
Amplifying specificity is higher.	Amplification specificity is lower.
Visualization of DNA product through: gel electrophoresis –turbidity – color change (SYBR Green I).	Visualization of DNA product is done through gel electrophoresis.
Does not require expensive thermo cycler	Require thermo cycler.

LAMP use of four separate primers, often designed to identify six distinct regions of the target gene. The following are the four primers that were used:

1. (FIP): Forward Inner Primer (FIP) consists of the F2 region (at the 3' end) that is complementary to the F2c region, and the same sequence as the F1c region at the 5' end.

2. F3 Primer (F3): consists of a F3 region which is complementary to the F3c region of the template sequence. This primer is shorter in length and lower in concentration than FIP.

3. (BIP): Backward Inner Primer (BIP) consists of the B2 region (at the 3' end) that is complementary to the B2c region, and the same sequence as the B1c region at the 5' end.

4. B3 Primer: Backward Outer Primer consists of a B3 region which is complementary to the B3c region of the template sequence.

Main point of primer design:

In LAMP, the quality of the primer design is very critical. Accounting for the positional base composition, GC contents and secondary structures formation above in primer regions can be defined following PrimerExplore (a special LAMP primer designing tool). The Nearest neighbor method can provide the Tm value.

The following is the main points of primer design.

1- Distance between primer regions

The distance between 5' end of F2 and B2 is considered to be 120-180bp, and the distance between F2 and F3 as well as B2 and B3 is 0-20bp.

The distance for loop forming regions (5' of F2 to 3' of F1, 5' of B2 to 3' of B1) is 40-60bp.

2. Tm value for primer regions

About 60-65°C in the case of GC rich and Normal, about 55-60°C for AT rich.

3.The stability of primer end

The dG calculated on 6bp from the following end regions should be less than -4kcal/mol, 5' end of F1c/B1c and 3' end of F2/B2 as well as F3/B3.

4.GC contents

About 50-60% in the case of GC rich and Normal, about 40-50% for AT rich.

5.Secondary structure

Primers should be designed so as not to easily form secondary structures. 3' end sequence should not be AT rich or complementary to other primers. (Zhao et al., 2016).

Steps of LAMP Procedure:

The LAMP reaction is done through various steps which begin with the annealing of the external primers (F3 and B3) to their binding sites on DNA. 1st, DNA polymerase activity of the strand displacement was initiated which also involves the other internal primers (FIP and BIP). This case of replacement reaction will lead to the formation of loops that would be engaged in other displacement reactions. This is a continuous amplification that will produce a large amount of amplified DNA at the endpoint of the reaction (within about one or two hours).

1.7 Brucellosis treatment

A multidrug regiment is commonly used in the treatment of brucellosis because monotherapy has a high rate of relapse. However, in vitro activity against *Brucella* has been demonstrated for many drugs; however, their clinical effectiveness is much lower. The cornerstone for the treatment of uncomplicated disease in adults and also children who are more than 8 years old is doxycycline, along with aminoglycoside (streptomycin or gentamicin) or rifampicin. Combined with rifampin, trimethoprim-sulfamethoxazole is a suitable substitute in children and also pregnant women where tetracyclines are contraindicated. The typical length of the uncomplicated disease treatment is 6 weeks.

1.8 Problem Statement:

Southern parts of Palestine have many more suspected *Brucella* infections reported by veterinary doctors of dairy animals. Usually, RBT is commonly used for the serological diagnosis of such cases. This serological test is unable to identify the cases of active infections, which are epidemiologically very significant and act as a source of infection. Moreover, the use of ELISA serological diagnostic tests continues to have these issues about specificity. as it is very cross-reactive with other Gram-negative bacterial genera. In addition, using ELISA for the detection of *Brucella* IgM antibodies is not very useful in the diagnosis of an active infection as this type of antibody survives even for weeks after the treatment.

With all such information considered above, we will undertake the development of DNA-based tests laudably known as LAMP for detecting live *Brucella* strains active in both the serum and milk or tissues drawn from animals. LAMP tests are considered a simple molecular approach that is field friendly method with high sensitivity and specificity since it is based on the use of six different primers that bind to the particular target DNA segment.

1.9 Objectives:

The main aim of the current study is to have a simple and standardized method for the detection of *Brucella* infection in milk and sera of diary animals, the specific objectives of the current study are:

1- To estimate the active incidence of brucellosis in diary animals in southern parts of Palestine based on serological methods.

2- To identify the exact bacterial species or subspecies that are the main infectious agents of brucellosis in our region based on PCR amplification of the 16s rRNA genes and its sequence analysis.

3- To develop a simple point of care (POC) test based on the sequence of the identified *Brucella* species and utilizing the LAMP technology.

4- To compare the developed LAMP based identification method with the most used serological methods for their usefulness in detection of active *Brucella* infections.

Chapter 2: Material and Methods

2.1 Sample Collection

A total of 500 serum samples were harvested from the sheep and goats mainly, as well as some cows and camels from different places in the southern parts of Palestine. Sample collection began in September 2019 but it also lasted until December the following year. This exercise involved taking between 3 and 5 ml of venous blood by a veterinarian, which is usually done upon request from the farm owners to ensure herd health or any other inspection purposes. Blood samples were allowed to stand for about an hour and this was immediately followed by the centrifugation of the clotted RBCs at 4000rpm. THE total number of positive results obtained by the Rose Bengal method is 200 samples which counts as a percentage of 4%. The serum samples were stored in 1.5 ml tubes at -20°C until the testing for confirmation and DNA extraction.

2.2 Serological testing: /Rose Bengal Test

The RBPT serological test was an agglutination assay specific for the qualitative and semi-quantitation of anti-*Brucella* antibodies in human as well as animal samples. The assay is made from the Rose Bengal *Brucella* Antigen (*Br. abortus* stain S99), which was suspended in a 1M lactate buffer and 5 g/L phenol at pH3.6, as well as other preservatives to stop bacterial growth It The RBT kit (Atlas Medical company) was also containing a positive control which is bovine serum containing anti-*Br. abortus* with a concentration of 50 IU/mL. Also a negative sera was included.

This test is defined by the quick agglutination procedure that occurs after the bonding of antibodies to *Br. abortus* bacterial cells. After mixing two equal volumes (about 50Âµl) of Rose

Bengal reagent with the tested samples, if a *Brucella* infection is present as a coloured agglutinate can be observed by the naked eye. The Company details were strictly followed according to the given procedure. We identically reanalyzed the positive cases to verify that finding.

2.3 DNA extraction from blood or serum samples by Phenol extraction method

It is possible that the total genomic DNA was extracted from the 200 clotted blood-collected serum samples. From each tested sample 300µl of serum or blood was mixed with 1: 1 volume of extraction lysis buffer (30µl). DNA extraction lysis buffer was made from (50mM NaCl, 10mM EDTA, and 50 m Tris-HCI p H7.4, 1% Triton X-100). Thirty microliters of 10mg/ml proteinase K (Sigma-Aldrich, St. Louis USA) were added to each mixture and incubated at 60 °C for two hours. After this incubation samples were removed and a 200 µl of phenol solution (pH 8) (Sigma Aldrich, St. Louis, USA) was added to each samples, followed by vortex for 1 min and centrifugation for another 3-5 minutes at 14000 rpm. The samples were re After centrifugation and the phase separation, The aqueous upper layer which contained DNA was removed using a micropipette to prevent the carryover of phenol solution into a new 1.5 ml Eppendorf tube. Since DNA was precipitated using 5M NaCl, this involved adding an additional amount of 12µl of 5M NaCl to make the final salt concentration to 0.2M NaCl. The DNA was then precipitated using 700µl of cold 100% ethanol molecular biology grade. All the samples were then refrigerated at -20ºC and stored overnight to allow for maximum DNA precipitation. on the next day DNA was recovered from the solution after centrifugation for 10 min at 14000 rpm at 4ºC, the supernatant was discarded and a small pellet was washed with 300µl of 70% ethanol, followed by centrifugation for 5 min at 14000 rpm at 4ºC, and allowed to air-dry to remove the remaining

alcohol. The samples were re-suspended in 100µl double distilled water (DDW) (Sambrook et al., 1989).

2.4 Polymerase Chain Reaction (PCR):

The PCR analysis was done using universal primers against a region in the DNA samples. The bacterial 16SrRNA (20 pmoles of 16SR and 20 pmoles of 16SD) primers as their sequence melting temperature is shown below in Table (4). DNA samples that had been tested positive for *Brucella* by serological tests underwent PCR analysis. The PCR reaction was carried out in a PCR tubes kit (Syntezza, Jerusalem) where each tube got a total volume of 25 microliters that included two primers (1 µl of each) and 5µl of the extracted DNA and the rest was 18µl of double distilled water (DDW).

In the case of bacterial DNA, the used thermal profile involved an initial denaturation step at 95°C that lasted for 5 min, followed by a complete denaturation at 95°C for 35 cycles each of 30 second, followed by annealing for 30 sec at 55°C TM, followed by full extension step (elongation) that involved 1 min at 72°C, and a final elongation step at 72°C for 10 min. (Green et al., 2012).

Table 4: 16S RNA PCR system that was used for DNA amplification from bacterial extracted DNA.

PCR system	**Primers**	**Tm (°C)**
16S rRNA	**Direct:16SD:**	**55°C**

	TCGTCGGCAGGGTCAGATGTGTATAAGA GACAGCGTACG **Reverse: 16SR:** GTGTCGTGGGCTCGGAGATGGTATTGTA TAAGAGACAGAGGAC	

2.5 Agarose Gel Electrophoreses:

The PCR products were visualized using a 1.5% agarose gel(1.5g agarose, 100ml 50X TAE and 10µl Ethidium bromide in 50X TAE electrophoresis running buffer (242g Tris base, 57.1ml glacial acetic acid and 100ml 0.5M EDTA (Ph8.0)). The Gene Ruler 50bp DNA ladder (Thermo Scientific, # SM0371) was used for sizing PCR amplified products (Sambrook et al., 1989).

2.6 LAMP assay

2.6.1 Primer design:

For this, two primer sets were developed also. In each LAMP assay, four primers were required to accomplish this reaction, these primers are: Two outer primers are custom-designed as the standard PCR annealing at the end of the target region. Two more primers called inner primers are designed for the amplification of internal regions both in the forward and reverse directions. These include the FIP and also BIP. Each of the inner primers consists of two oligonucleotides connected with a sequence of TTTT. Every internal primer binds to the sense and antisense strands of the target DNA near the external primers. In the first two steps of LAMP, all four primers are used; in cycling reaction during strand displacement DNA synthesis, only inner primers are utilized. In this latter step, the stem and loop amplification products are created. The outer primers of the LAMP assay will be just like those used in PCR (Figure 3). A

primer design program for LAMP is available on the market called (PrimeExplorer-Fujiitsu, Japan). In our case, we designed the following primers manually, and each of them needs to have a very high melting temperature (higher than 60^{o}C).

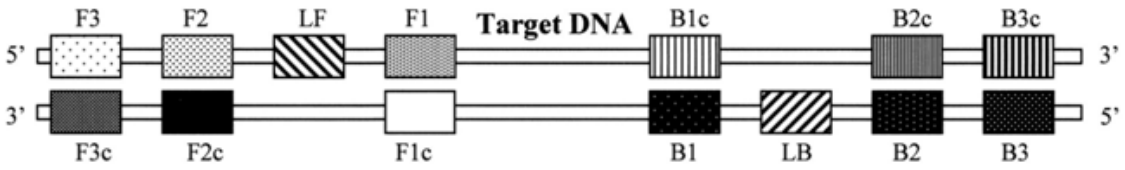
More specifically, two sets of primers were designed for the *Brucella* LAMP assay; each set was based on a 400 bp region from the *Brucella* 16S rDNA gene segment. For this, initially, The complete 16S rDNA gene sequence (which is about 1400 bp) from several bacterial species were downloaded from the GenBank and aligned to see the similar regions between these bacterial species and to avoid cross-amplification. Table 5 shows the shows primer sequences that were used for this purpose and the accession numbers from the GenBank of the 16S rDNA gene, these bacterial species were chosen to be the most closely related to *Brucella spp* and other bacterial species that may be in blood or intracellular. Appendix 1, shows DNA alignment of all used 16S rDNA genes. these bacterial species are considered to be the closest relatives to *Brucella spp*. together with the other bacteria genera in the blood or intracellular tissues where Brucellae can be found. Appendix 1 presents the DNA alignment for all of the mentioned 16S rDNA genes. Based on this alignment it was concluded the two less shared regions among all aligned sequences were two region in this gene (16S rDNA), a- the first region was from 1-300 bp, and b- the second region was from 930-1400 bp region. Simply the first 300 bp and the last 400 bp of the 16S rDNA gene was used for this primers design. Figure 3, shows the sequence of the two selected regions of *Brucella* bacterial 16S rDNA genes with marked primers sites that were selected according to primer guide in figure 4. Also table 5, shows the primer sequences of the two LAMP sets.

***Brucella* 16 sRNA gene:**

```
   1 catggctcag aacgaacgct ggcggcaggc ttaacacatg caagtcgagc gccccgcaag
  61 gggagcggca gacgggtgag taacgcgtgg gaacgtacca tttgctacgg aataactcag
 121 ggaaacttgt gctaataccg tatgtgccct tcgggggaaa gatttatcgg caaatgatcg
 181 gcccgcgttg gattagctag ttggtggggt aaaggctcac caaggcgacg atccatagct
 241 ggtctgagag gatgatcagc cacactggga ctgagacacg gcccagactc ctacgggagg
 301 cagcagtggg gaatattgga caatgggcgc aagcctgatc cagccatgcc gcgtgagtga

 901 accttaccag cccttgacat cccggtcgcg gttagtggag acactatcct tcagttaggc
 961 tggaccggag acaggtgctg catggctgtc gtcagctcgt gtcgtgagat gttgggttaa
1021 gtcccgcaac gagcgcaacc ctcgccctta gttgccagca ttcagttggg cactctaagg
1081 ggactgccgg tgataagccg agaggaaggt ggggatgacg tcaagtcctc atggccctta
1141 cgggctgggc tacacacgtg ctacaatggt ggtgacagtg ggcagcgagc acgcgagtgt
1201 gagctaatct ccaaaagcca tctcagttcg gattgcactc tgcaactcga gtgcatgaag
1261 ttggaatcgc tagtaatcgc ggatcagcat gccgcggtga atacgttccc gggccttgta
1321 cacaccgccc gtcacaccat gggagttggt tttacccgaa ggcgctgtgc taaccgcaag
1381 gaggcaggcg accacggtag ggtcagcgac tggggtgaag tcgtaacaag
```

Figure 3: *Brucella* 16S rRNA DNA sequence, showing the sites that were used for primer design of LAMP system 1 and LAMP system 2.



FIP (Forward Inner Primer)= 5'-F1c/TTT/F2
BIP (Backward Inner Primer)= 5'-B1c/TTT/B2
F3 Primer= 5'-F3
B3 Primer=5'-B3

Figure 4: Representative sketch showing the way that used as a guide to construct LAMP primers.

Table 5: DNA sequence of LAMP primers set 1 and set 2

Primer set 1:
F3=catggctcagaacgaacgct
B3=ctctcacgcggcatggctg
FIP=ctgccgcccttgcggggc/TTT/ggcttaacacatgcaag
BIP=gggaggcagcagtgggg/TTTT/ggttcgccctaagtcc

Primer set 2:
F3=gtggagacactatcct tcag
B3=acgacttcaccccagtc
FIP=ctgacgacagccatgcagcacc/TTT/gttaggctggaccggag
BIP=ggcgctgtgctaaccgcaag/TTTT/ccctaccgtggtcgcctgcc

2.6.2 LAMP Reaction:

LAMP reaction mixtures was carried out in a total of 25ul reaction mixture containing 5 ml of the template DNA, 40pmol internal primers, 5pmol of external primers, 8U of Bst DNA polymerase large fragment (New England Bio-labs Inc, MA, USA), 1.4mM dNTPs and reaction buffer (containing 20 mM Tris-HCl pH 8.8, 10mM KCl, 10 mM (NH4)2SO4, 8mM MgSO4, 0.1% Triton-X was used. The reaction was carried out at 65oC for 1-2 hours (Parida et al., 2008).

2.6.3 LAMP Amplicon detection and analysis:

Analysis and detection of the amplified products was done by the following methods. 1- simply by agarose gel electrophoresis to show amplification on gel that was represented by multiband. and 2- detection of LAMP amplified products was performed using SYBR Green I stain (Invitrogen, Carlsbad, CA). This was done by the addition of One µl of SYBR Green I to each reaction after the incubation period, so a positive LAMP amplification was seen directly by eye, as the color of the reaction solution changed from orange to green in the presence of high concentration of DNA that represents LAMP amplification reactions.

2.6.4 LAMP amplification and Optimization conditions:

Amplification temperatures between 60ºC and 65ºC was examined for optimal amplification in experiments amplifying different concentrations of DNA of *Brucella* bacterial DNA. The standard LAMP reaction was tested against two temperatures (60 ºC and 65 ºC), and this reflects the highest and the minim used temperatures for any LAMP assay.

2.7 DNA PCR purification

To remove phenol remnants and other LAMP reaction inhibitors, PCR products were purified by GeneJET PCR purification Kit (Thermo Scentific, USA), according to manufacturer's instructions with some modifications. The DNA purification steps were done as following: The remaining quantity of PCR product for each sample was added into eppendorf tube; 100µl of sterile D.D.H2O then was added. Next, 200µl of binding buffer was added in each tube and centrifuged for 60 sec to discard flow- through. 700µl wash buffer (ethanol) was added to the GeneJET purification column for each sample. Then each tube was centrifuged for 3 min and the flow-through was discarded. GeneJET purification column was centrifuged again to remove any residual wash buffer. The GeneJET purification column next was transferred into 1.5ml microcentrifuge tube and 30µl D.D.H2O was added to concentrate DNA. Then, all tubes were incubated at RT for 2 min and then centrifuged for 60 sec.

2.8 Data analysis

In this work, we investigated the data mostly using Inferential analysis, a type of data analysis. We also used another type of data analysis, which was diagnostic analysis.

Chapter 3: Results

3.1 Serological examination (Rose-Bengal *Brucella* agglutination test):

Out of the 5000 total samples collected within a period of two years to be tested for Brucellosis, only 20s were found positive. This is a very straightforward agglutination test with either positive or negative outcomes.

3.2 PCR amplification of serum/blood extracted DNA:

After extracting DNA from the positive samples, we amplified the DNA of bacteria using a segment from the 16S rRNA gene (approximately 450 bp). The produced products were sent for DNA sequence analysis, but no results were obtained to confirm infection by *Brucella*.

3.3 LAMP assay:

The two designed LAMP primer systems (namely called LAMP set 1 and LAMP set 2), were tested against 1 ng and 0.01 ng of total genomic DNA of *Br. abortus*. The reaction was first carried at 63^{o}C for both set of primers, taking into account that the optimal temperature used for LAMP reaction is between 60^{o}C to 65^{o}C, and this is the optimal temperature of Bst DNA polymerase I enzyme. Figure 5 shows agarose gel electrophoresis of the two produced amplicons using these two sets of primers. It is clearly seen that LAMP primers set 2 showed a better amplification (more sensitive), than LAMP primers set 1.

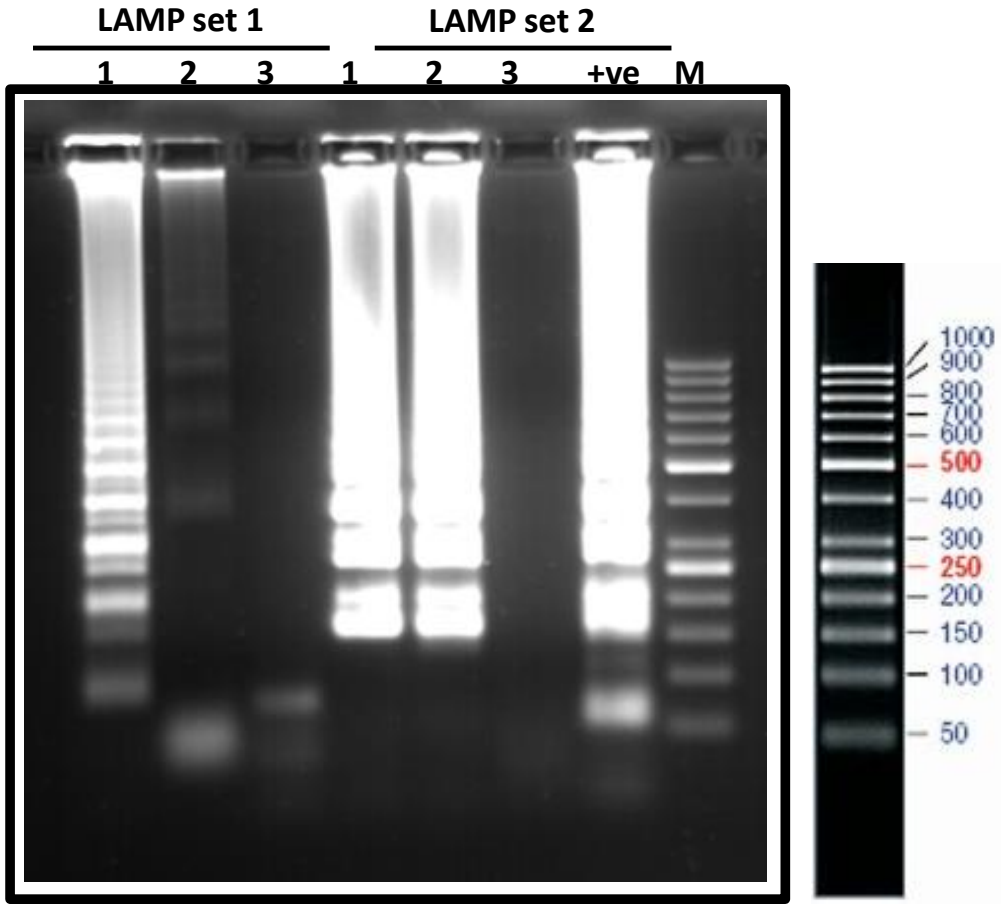


Figure 5: Agarose gel electrophoresis showing the amplification profile using the two designed sets of LAMP primers. 1= 1ng of DNA, 2= 0.1ng of DNA, 3= negative control.

3.4 LAMP reaction optimization:

The two LAMP primers sets were tested at two different of reaction temperature (60^{o}C and 65^{o}C). This was done to see if at lower temperature it is possible to have a more sensitive amplification than at 65^{o}C. The reactions were carried using three different DNA concentrations of 100 different magnitude. The following DNA concentrations were used (1ng, 0.01ng, and 1fg). Figure 6 shows that the amplification was more sensitive at 60^{o}C compared to that at 65^{o}C, similar results was obtained for the both sets of LAMP primers. It is known that at lower amplification temperature (less than 60^{o}C) the primers may do non-specific amplification, so all the coming reactions were carried at 63^{o}C which is the initial tried temperature and that still could give a sensitive amplification.

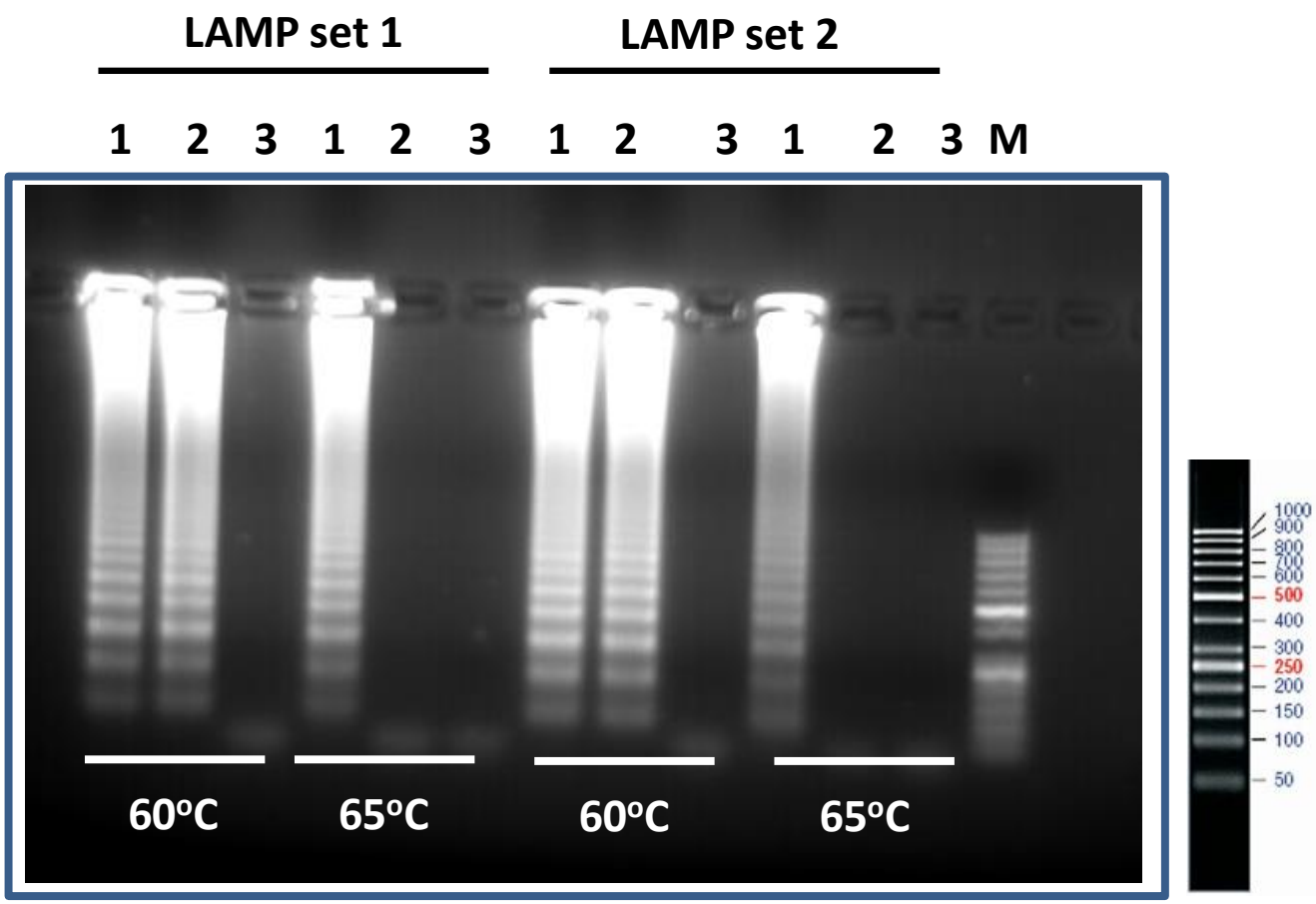


Figure 6: Optimization conditions for the two designed LAMP set primers using two different temperature reactions (60°C and 65°C). 1=1ng, 2=0.01ng, 3=1fg, M: DNA size marker.

3.5 Sensitivity of LAMP reactions using the two sets of primers:

Sensitivity test was performed for the two LAMP sets of primers both were carried at 63^{o}C using 10 fold dilution of *Br. abortus* genomic DNA. The used DNA concentrations were (1ng, 0.1ng, 0.01ng, 1fg, 0.1fg, and No DNA negative control). As can be seen from figure 7 it was possible to have a strong amplification reactions using the two sets of primers, but it was clearly seen also that LAMP primer set 2 were more sensitive with one magnitude of DNA concentration compared to LAMP primers set 1. Based on that, the results in the primer set 2 were better than the primer set 1, we decided to use only LAMP primer set 2 for the coming experimental work.

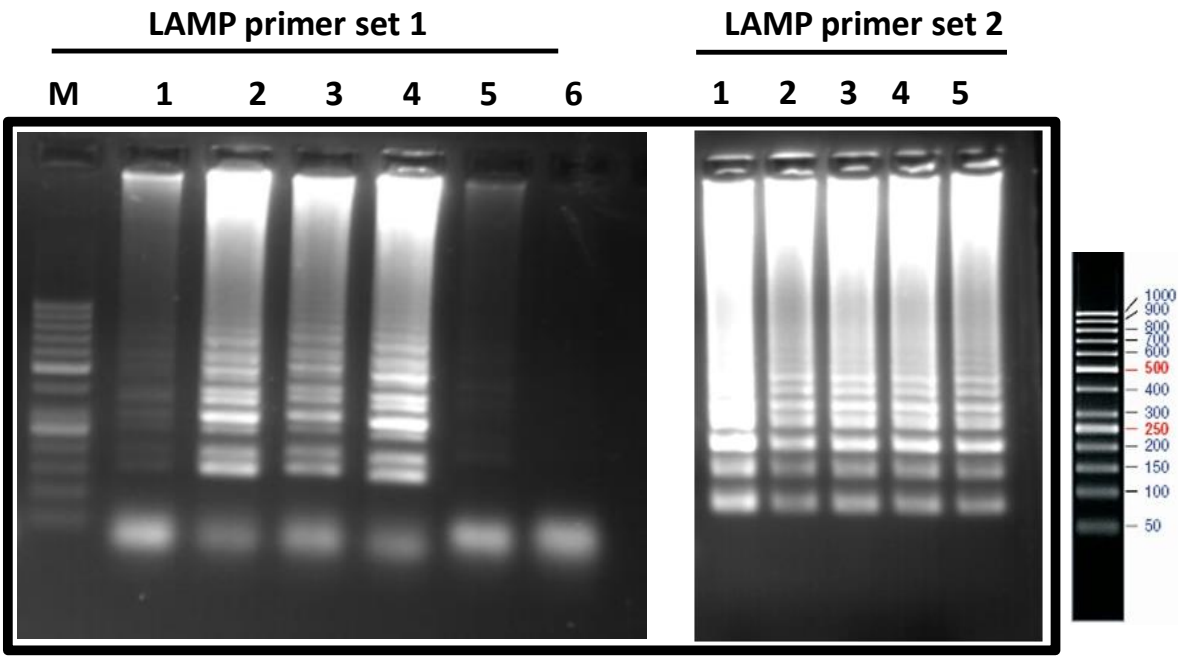


Figure 7: LAMP sensitivity test, Agarose gel electrophoresis for both LAMP primers set 1 and set 2 using different DNA concentrations. LAMP reaction was set at 60°C. 1-1ng, 2-0.1ng, 3-0.01ng, 4-1fg, 5-0.1fg, 6- No DNA control, M-size marker

3.6 LAMP specificity test:

This test was performed to examine the specificity of the potential used LAMP primers. Only LAMP primers set 2 was used in this test, since it was more sensitive compared to LAMP primers set 1. So, LAMP reaction including set 2 primers were tested against 1ng of genomic DNA on the following bacterial species (1- *Agrobacterium*, 2- *Escherichia coli*, 3- *Pseudomonas*, 4- *Bacillus subtilis*, 5-*Staphylococcus aureus*, 6- *Brucella abortus*, 7- negative control(no DNA) (Figure 8).

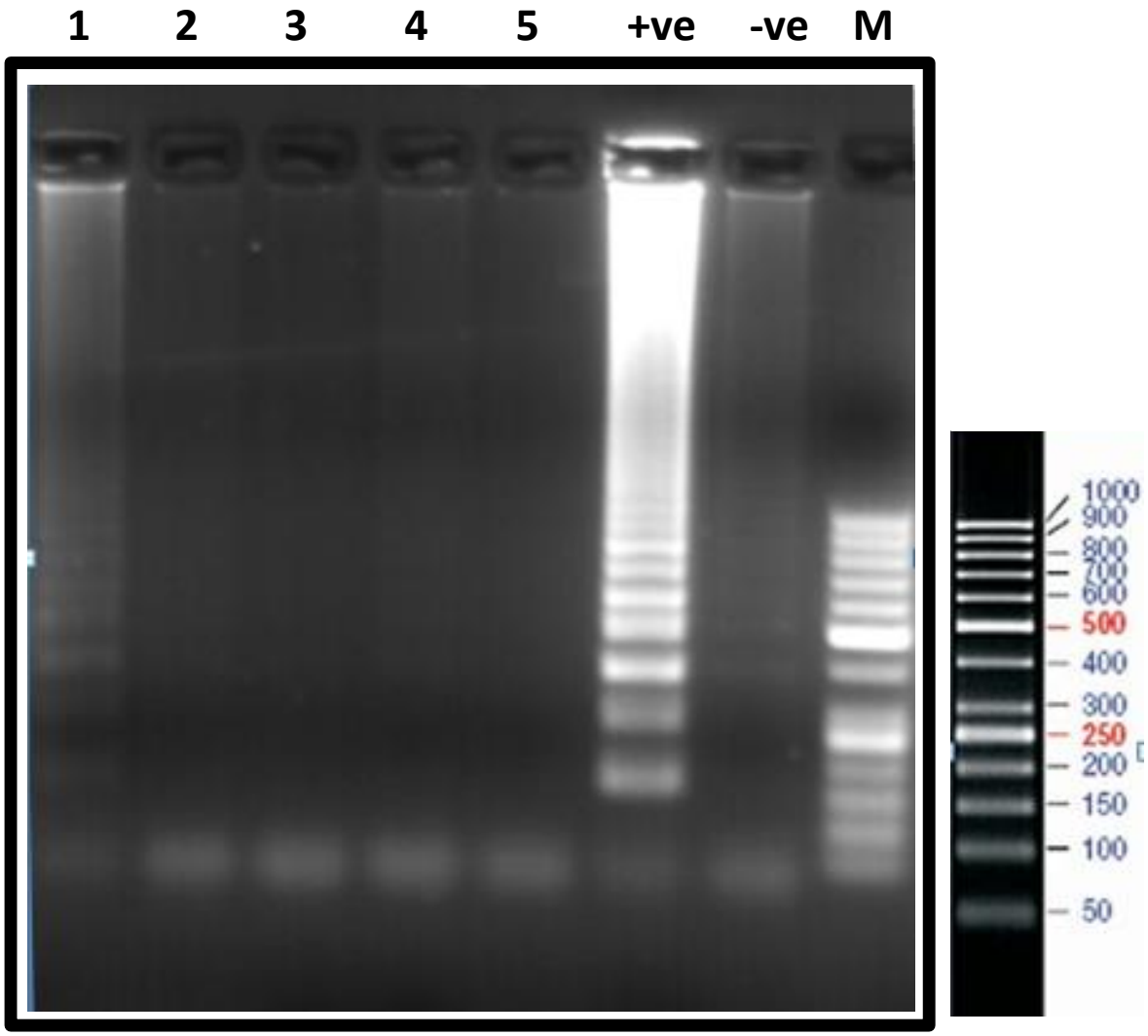


Figure 8: Specificity test for LAMP primers set 1. Agarose gel electrophoresis showing DNA amplification targeting 1ng of different bacterial species. 1- Agrobacterium, 2- Escherichia coli, 3- Pseudomonas, 4- Bacillus subtilis, 5-Staphylococcus aureus, 6- *Brucella* abortus, 7- negative control (no DNA).

3.7 Visual detection of amplified LAMP products:

In this part of the work two detection methods were tested, namely they are the agarose gel electrophoresis and visual detection by the use of SYBR Green I stain. As it was indicated in material and methods the addition of SYBR Green I stain to a positive LAMP reaction (that is characterized by having large concentration of amplified DNA) will cause a color change from orange (the normal SYPBR Green I color) to yellow and this indicates a positive amplification reaction. This method was compared to the agarose gel electrophoresis and similar results were obtained by the two reaction (Figure 9). It was clearly seen that the sensitivity of the SYPR Green I is proportionally related to the agarose electrophoresis analysis, as there is a bands that can be seen after agarose electrophoresis analysis step; then it is possible to see a change in color to yellow indicating a positive reaction. So it is possible only to detect positive amplification by the addition of SYPR Green I to the LAMP reaction.

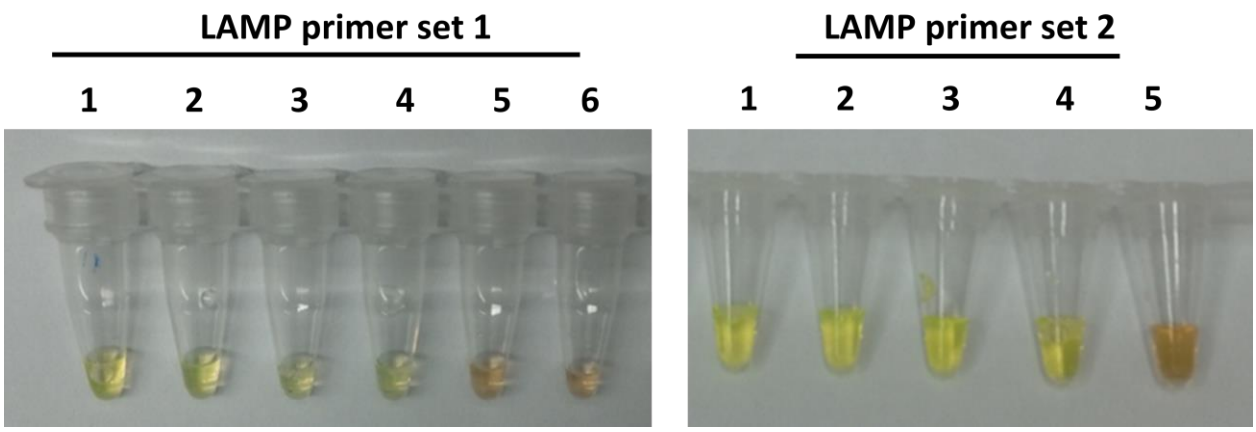


Figure 9: Visual detection of amplified LAMP products using SYBR Green I stain. The change from orange to Yellow indicates a positive reaction. 1-1ng, 2-0.1ng, 3-0.01ng, 4-1fg, 5-0.1fg, 6- No DNA control.

3.8 Sample testing using LAMP primers set 1:

Random samples of DNA extracted from positive *brucella* cases (approved by rese-bengal test), were tested by LAMP method using primers set1. At the beginning 10 samples were used for this test. The samples were composed of DNA extracted from blood samples of positive brucellosis cases, these samples gave a negative results using the LAMP procedure at it optimized conditions. Many other samples were tested as well and most of them gave a negative results (Figure 10 A). From previous studies we noticed the inhibition behavior of LAMP test reactions that target DNA amplified using phenol based method (Opel et al., 2010). For this reason, 10 of the tested samples that gave a negative reaction were purified by DNA PCR purification kit to remove inhibitors and to obtained a high quality DNA material. LAMP testing of the cleaned DNA samples showed an increase in number of positive LAMP amplified reactions (Figure 10 B). This results indicates the importance of inhibitor removal in each time and before applying LAMP reaction test.

40 samples extracted DNA and cleaned were tested using the LAMP technique, and only 26 of these samples showed positive results after DNA purification and inhibitor removal. Figure 11 shows 30 of these randomly selected samples that were subjected to the LAMP test. Figure 12 also shows some of the samples that were observed using SYBR Green I staining.

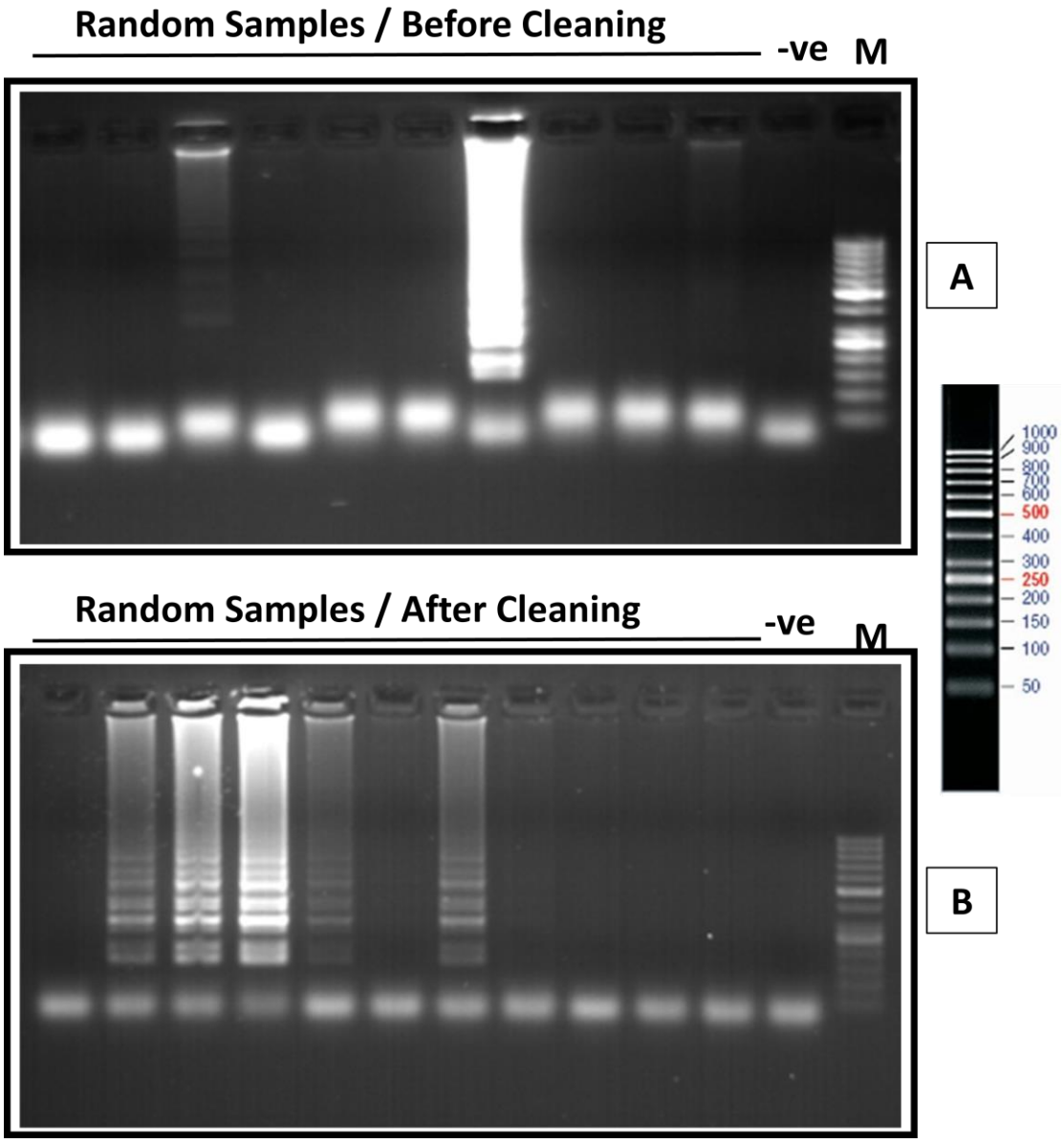


Figure 10: Randomly selected samples (10 samples + negative control) that were tested by LAMP primers. A: Extracted DNA by phenol method. B: Extracted DNA by phenol method and purified by PCR purification kit.

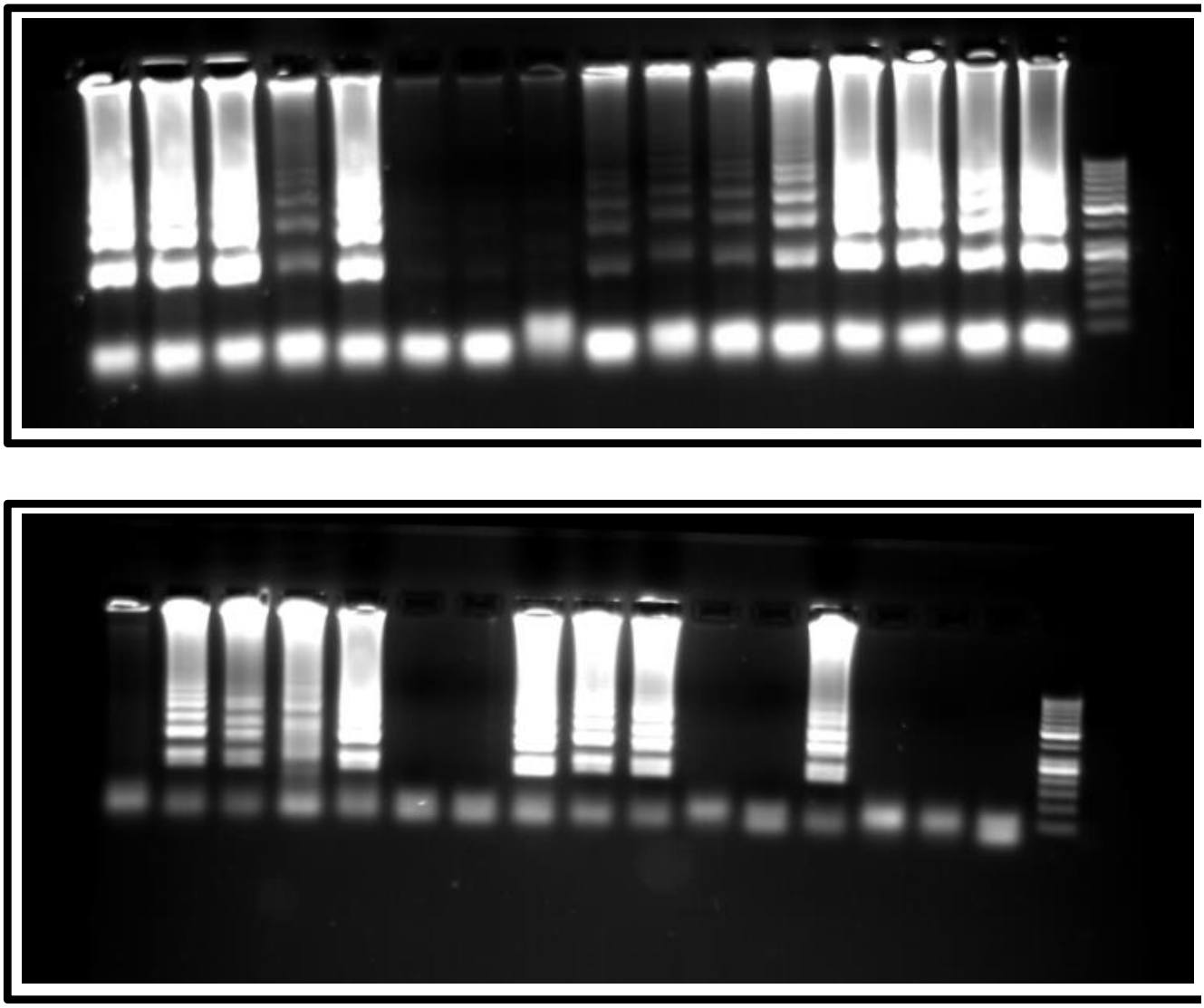


Figure 11: Agarose gel electrophoresis analysis of a randomly selected samples. The first 30 reaction are DNA extracted from *Brucella* positive cases by Rose-Bengal agglutination test and after their purification by PCR purification kit. The last two lanes are negative controls.

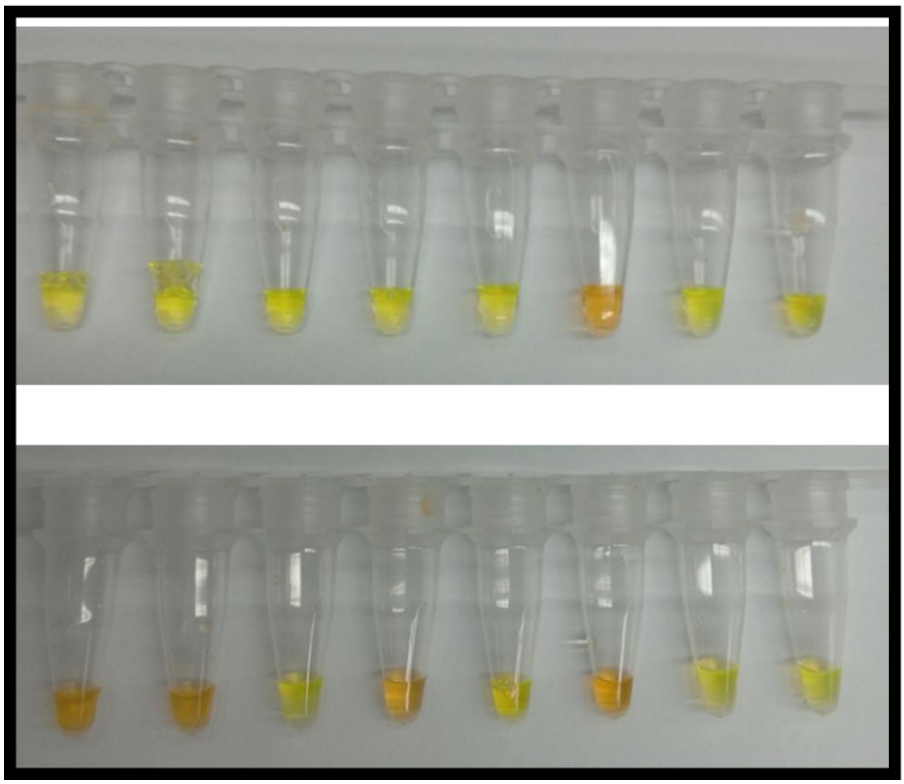


Figure 12: SYBR Green I staining of a randomly selected samples that were amplified by LAMP primers set 1.

Chapter 4: Discussion

RBPT test is the most commonly used of all the tests in *Brucella* diagnosis. The basis of this test usually depends on the agglutination of formalin fixed bacterial cells that will clump in a response to their reactivity with anti *Brucella* antibodies which are present in animals or even human body. The test is comparable to the most commonly used agglutination tests that only react positively with an advanced brucellosis infection and elevated antibody concentration. Therefore, several of the positive cases will be presented as false negatives. The agglutination tests are known as rapid tests and they can detect the results in a very short time. RBPT test is also has cross reactivity with some other bacterial infections such as: *Rickettsia, Neisseria, Pseudomonas, Bacillus* and *Mycoplasma* are some of the bacteria associated with blood infections that are intracellular; these symptoms mimics *Brucella* infection (Bonfini et al., 2018).

The RBPT is a slide-type agglutination assay that uses a stained *Br. abortus* suspension and plain serum at pH 3.6–3.7, It could be used as a screening method for human brucellosis because of its simplicity and it is also appropriate in small laboratories where resources are limited. But, due to the ambiguity concerning the test worthiness current WHO recommendations state that RBPT findings should be confirmed through other experiments (Corbel, 2006). Many tests have been commonly employed in the serological validation of brucellosis, implying the lack of a perfect technique. A suspension of *Br. abortus* in an acid buffer is used in the RBPT test. It can distinguish between agglutinating and nonagglutinating antibodies and prevents the prozone effect. Therefore, the rose Bengal test is very sensitive for diagnosing the *Brucella spp*. infection, regardless of the stage of the disease.

In the 2020, some researchers carried out a statistical analysis. 16 previous studies were used to rate the sensitivity and specificity of RBPT. However, they noted that only six of the

sixteen papers reviewed were considered to be RBPT with a high or moderate scientific quality and addressed further. Ten of the sixteen studies were deemed to be low on the scientific quality in terms of the data provided (Ekiri et al., 2020). Although some studies have shown the rose Bengal test to be a high specificity in endemic areas, there is a lack of information regarding its sensitivity. However, the results are also biased towards healthier individuals or the patients with other diseases in the majority of trials. The proportion of patients who were exposed to brucellosis at work or those who recently went through the disease is disproportional within the control groups (Moreno et al., 1992).

In the current study, 4 % of Brucellosis was found in the infected sheep and lacked approval through other serological tests rather than agglutination test; However, we intended to verify this result using DNA detection on positive cases' blood. This was achieved through DNA extraction and PCR amplification of the bacterial species available using 16S rRNA gene. The general length of the rRNA gene for most bacterial species is approximately 1400 bp, and it consists about nine variable regions with various degrees among different cell lines. The primers that were used to the current classical PCR came from the V3/V4 region. This part of the 16S rRNA region has a conserved segment from which the universal primers were derived and are employed to amplify a variable portion that is sequenced so as to incorporate both sequences for identification purpose. We managed to PCR amplify the 450 bp region of bacterial 16S rRNA with universal primers; however, we did not have the sequence data in order to confirm cellular positive *Brucella* cases by RBT.

In this study we developed a LAMP assay, which enables DNA amplification and product detection in field laboratories. LAMP utilizes an auto-cycling strand displacement DNA synthesis using (Bst) DNA polymerase known to have a high strand-displacement activity, (Bst) and four

specifically designed primers that allow the amplification of products having stem – loop structures. The use of an amplification system with a number of primers is therefore anticipated to be very specific based on the fact that it has already been shown for different infectious agents (viral, bacteria and parasites). This technique can increase several copies of the target DNA sequence to 10^9 copies within less than an hour under the isothermal conditions and with a very high degree of specificity. LAMP can be easily modified to suit the low-tech field laboratories and may not require DNA decontamination. The set of 4 primers needed for LAMP can be designed based on the bacterial 16S rRNA.

The newly emerging novel technique is LAMP a very elementary, gene amplification procedure carried out under the conditions of constant temperature. Although it may be very rudimentary, its precision and sensitivity render the unnecessary use of a thermal cycler. This method differs from PCR (Mori and Notomi, 2009) because of the very high sensitivity and specificity. Additionally, the advanced tools were not necessary and the effect was proven in a relatively short period of time with only a simple heater. Despite being unnecessary for this specific procedure, the sensitivity and accuracy of such high-performance are so good that it can be performed using a dry plate (Notomi et al., 2001). It could be obtained by combining SYBR Green 0.1% into the result in order to identify it under UV-light, with no need for any post PCR processes like electrophoresis. Since phenotype is a faster and more accurate than other molecular diagnostics departments, the fast speed and accuracy have given priority to this method even in clinical laboratories. Replacement techniques are often very required and also implemented as a matter of course.

This approach can definitely aid in the prevention of infection. Molecular techniques are now highly sensitive and also specific enough to detect the *Brucella*. The outcome of this study

can be used to help reduce the occurrence of infection and also cost avoidance by early diagnosis to treat brucellosis with appropriate patient care. *Brucella* diagnosis without the expensive high-end equipment, accurate sensitivity and specificity due to the feasibility of LAMP technique in all clinical services would be possible with a modest budget in not too long time.

There are several features that contribute to the reliability of LAMP-assays feasible in low tech labs available. First, the amplification can be initiated with double stranded DNA, and since everything is subsequently performed at the same temperature it does not require more than an incubator or a water bath, which are typical equipment widely available in basic laboratories; Secondly, detection of reaction products also likely to be relatively straightforward using nucleic acid dye SYBR GREEN 1 that changes color from orange into green. In addition, extraction methods suited the major costs pertain to the primers, nucleotides and also DNA polymerase used in the amplification reaction. These regents, though, could be provided as preassembled kits and can undergo a long-term storage in a low-tech laboratory.

Conclusion

In the case of LAMP, let us analyze its benefits compared to other available methods as we can see simplicity accuracy and also timely nature integrated in this method. When the right brucellosis diagnosis is carried out easily. Thus, the developed test could serve as a rapid one for the proper diagnosis of brucellosis from blood specimens. Using SYBR Green I in this test can prevent the time-consuming analysis by agarose gel electrophoresis. Besides pinpointing *Brucella*. It increases the effectiveness of the treatment whilst also lessening antibiotic use and minimizing costs. Misdiagnosis gives rise to inappropriate treatment.

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Appendix 1: 16sRNA DNA alignment:

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Brucella        -----------catggctcagaacgaacgctggcggcaggcttaacacatgcaagtcgag         49
Rickettsia      -----------cctggctcagaacgaacgctatcggtatgcttaacacatgcaagtcgaa         49
Mycoplasma      ------------------------------ctggcggcatgcctaatacatgcaagtcgaa         31
Neisseria       ------------------------------------------------------------         0
Klebsiella      agagtttgaatcatggctcagattgaacgctggcggcaggcctaacacatgcaagtcgag         60
Pseudomonas     -----tttgatcctggctcagattgaacgctggcggcaggcctaacacatgcaagtcgag         55


Brucella        cgc-----------------cccgc--aaggggagcggcagacgggtgagtaacgc-gtgg         90
Rickettsia      cggattaattagagcttgctctag--ttaattagtggcagacgggtgagtaacac-gtgg         106
Mycoplasma      cggaggtgcttgcac--------------ctcagtggcgaacgggtgagtaacacgtatc         77
Neisseria       -cagcacagagaagcttgcttctcgggtggcgagtggcgaacgggtgagtaacat-atcg         58
Klebsiella      cggtagcacagagagcttgctctcgggtgacgagcggcggacgggtgagtaatgt-ctgg         119
Pseudomonas     cggatgaagggagc----ttgctcctggattcagcggcggacgggtgagtaatgc-ctag         110
                                        ** ***  ************


Brucella        gaacgtaccatttgctacggaataactcagggaaacttgtgctaataccgtatgtgccct         150
Rickettsia      gaatctacccatcagtacggaataacttttagaaataaaagctaataccgtatattctct         166
Mycoplasma      taacctacctcatagcgggggataacttttggaaacgaaagataataccgcatgtagatc         137
Neisseria       gaacgtaccgagtagtgggggataactgatcgaaagatcagctaataccgcatacgtctt         118
Klebsiella      gaaactgcctgatggaggggataactactggaaacggtagctaataccgcataaygtcg         179
Pseudomonas     gaatctgcctggtagtgggggataacgtccggaaacgggcgctaataccgcatacgtcct         170
                 **  * **          ** *****     ****     * ******** **


Brucella        ------------------------tcgggggaaagatttatcggcaaatgatcggcccgcg         187
Rickettsia      ------------------------acggaggaaagatttatcgctgatggatgggcccgcg         203
Mycoplasma      ttattatcgcatgagaaaagatcaaaagaaccgtttggttcactatgagatggggatgcg         197
Neisseria       ---------gagagagaaagcaggggactttcgggccttgcgctattcgagcggccgata         169
Klebsiella      ---------caagaccaaagtgggggaccttcgggcctcatgccatcagatgtgcccaga         230
Pseudomonas     ---------gagggagaaagtgggggatcttcggacctcacgctatcagatgagcctagg         221
                                                              **   *


Brucella        ttggattagctagttggtggggtaaaggctcaccaaggcgacgatccatagctggtctga         247
Rickettsia      tcagattaggtagttggtgtggtaatggctcaccaagccgacgatctgtagctggtctga         263
Mycoplasma      gcgtattagctagtaggtgagataatagcccacctaggcgatgatacgtagccgaactga         257
Neisseria       tctgattagctagttggtggggtaaaggcccaccaaggcgacgatcagtagcgggtctga         229
Klebsiella      tgggattagctagtaggtggggtaacggctcacctaggcgacgatccctagctggtctga         290
Pseudomonas     tcggattagctagttggtggggtaaaggcctaccaaggcgacgatccgtaactggtctgg         281
                    ***** **** **** * ***  **  *** ** *** ***   ** * *  ***


Brucella        gaggatgatcagccacactgggactgagacacggcccagactcctacgggaggcagcagt         307
Rickettsia      gaggatgatcagccacactgggactgagacacggcccagactcctacgggaggcagcagt         323
Mycoplasma      gaggttgatcggccacattgggactgagatacggcccagactcctacgggaggcagcagt         317
Neisseria       gaggatgatccgccacactgggactgagacacggcccagactcctacgggaggcagcagt         289
Klebsiella      gaggatgaccagccacactggaactgagacacggtccagactcctacgggaggcagcagt         350
Pseudomonas     -aggatgatcagtcacactggaactgagacacggtccagactcctacgggaggcagcagt         340
                 *** *** * * **** *** ******* **** ************************


Brucella        ggggaatattggacaatgggcgcaagcctgatccagccatgccgcgtgagtgatgaaggc         367
Rickettsia      ggggaatattggacaatgggcgaaagcctgatccagcaataccgagtgagtgatgaaggc         383
Mycoplasma      agggaatttttcacaatggacgaaagtctgatgaagcaatgccgcgtgagtgatgacggc         377
Neisseria       ggggaattttggacaatgggcgcaagcctgatccagccatgccgcgtgtctgaagaaggc         349
Klebsiella      ggggaatattgcacaatgggcgcaagcctgatgcagccatgccgcgtgtgtgaagaaggc         410
Pseudomonas     ggggaatattggacaatgggcgaaagcctgatccagccatgccgcgtgtgtgaagaaggt         400
                 ****** **  ******* ** *** *****  *** ** *** ***  *** ** **


Brucella        cctagggttgtaaagctctttcaccggtgaaga-------------------------ta         402
Rickettsia      cttagggttgtaaagctcttttagcaaggaaga-------------------------ta         418
Mycoplasma      cttcgggttgtaaagctctgttgtaagggaagaaaaaataaagtaggaaatgactttatc         437
Neisseria       cttcgggttgtaaaggacttttgtcagggaagaaaaggctgttgctaataccagcggctg         409
Klebsiella      cttcgggttgtaaagcactttcagcggggaggaaggcgwtaaggttaataacctyrkcga         470
Pseudomonas     cttcggattgtaaagcactttaagttgggaggaagggcagtaagttaataccttgctgtt         460
                * * ** ********  ** *       ** **


Brucella        atgacggtaaccggagaagaagccccggctaacttcgtgccagcagccgcggtaatacga         462
Rickettsia      atgacgttacttgcagaaaaagccccggctaactccgtgccagcagccgcggtaagacgg         478
Mycoplasma      ttgacagtaccttaccagaaagccacggctaactatgtgccagcagccgcggtaatacat         497
Neisseria       atgacggtacctgaagaataagcaccggctaactacgtgccagcagccgcggtaatacgt         469
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Klebsiella      ttgacgttacccgcagaagaagcaccggctaactccgtgccagcagccgcggtaatacgg        530
Pseudomonas     ttgacgttaccaacagaataagcaccggctaacttcgtgccagcagccgcggtaatacga        520
                 ****  **       *  ****  *********  ******************* **


Brucella        agggggctagcgttgttcggatttactgggcgtaaagcgcacgtaggcggacttttaagt        522
Rickettsia      agggggctagcgttgttcggaattactgggcgtaaagagtgcgtaggtggtttagtaagt        538
Mycoplasma      aggtggcaagcgttatccggatttattgggcgtatagggtgcgtaggcggttttgcaagt        557
Neisseria       agggtgcgagcgttaatcggaattactgggcgtaaagcgggcgcagacggttacttaagc        529
Klebsiella      agggtgcaagcgttaatcggaattactgggcgtaaagcgcacgcaggcggtctgtcaagt        590
Pseudomonas     agggtgcaagcgttaatcggaattactgggcgtaaagcgcgcgtaggtggttcagcaagt        580
                ***  ** ******   **** *** ******** ** *  ** **  **      ***


Brucella        caggggtgaaatcccggggctcaaccccggaactgcctttgatactggaagtcttgagta        582
Rickettsia      tggaagtgaaagcccggggcttaacctcggaattgctttcaaaactactaatctagagtg        598
Mycoplasma      ttgaggttaaagtccggagctcaactccggt-tcgccttgaaaactgtattactagaatg        616
Neisseria       aggatgtgaaatccccgggctcaacccgggaactgcgttctgaactgggtgactcgagtg        589
Klebsiella      cggatgtgaaatccccgggctcaacctgggaactgcattcgaaactggcaggctagagtc        650
Pseudomonas     tggatgtgaaatccccgggctcaacctgggaactgcatccaaaactactgagctagagta        640
                  *  ** ***  ** * *** ***   **    ** *     ***      ** ** *


Brucella        tggtagaggtgagtggaattccgagtgtagaggtgaaattcgtagatattcggaggaaca        642
Rickettsia      tagtaggggatgatggaattcctagtgtagaggtgaaattcttagatattaggaggaaca        658
Mycoplasma      caagagaggtaagcggaattccatgtgtagcggtgaaatgcgtagatatatggaagaaca        676
Neisseria       tgtcagagggaggtagaattccacgtgtagcagtgaaatgcgtagagatgtggaggaata        649
Klebsiella      ttgtagaggggggtagaattccaggtgtagcggtgaaatgcgtagagatctggaggaata        710
Pseudomonas     cggtagagggtggtggaatttcctgtgtagcggtgaaatgcgtagatataggaaggaaca        700
                    ** **      ***** *  ******  ******* * **** **  * * *** *


Brucella        ccagtggcgaaggcggctcactggaccattactgacgctgaggtgcgaaagcgtggggag        702
Rickettsia      ccggtggcgaaggcggtcatctgggctacaactgacgctgatgcacgaaagcgtggggag        718
Mycoplasma      cctgtggcgaaagcggcttactggcttgttattgacgctgaggcacgaaagcgtggggag        736
Neisseria       ccgatggcgaaggcagcctcctgggacaacactgacgttcatgcccgaaagcgtgggtag        709
Klebsiella      ccggtggcgaaggcggccccctggacaaagactgacgctcaggtgcgaaagcgtggggag        770
Pseudomonas     ccagtggcgaaggcgaccacctggactgatactgacactgaggtgcgaaagcgtggggag        760
                **  ******* **      ****      * ****  * * *  ************ **


Brucella        caaacaggattagataccctggtagtccacgccgtaaacgatgaatgttagccgtcgggg        762
Rickettsia      caaacaggattagataccctggtagtccacgccgtaaacgatgagtgctagatatcggag        778
Mycoplasma      caaataggattagataccctagtagtccacgccgtaaacgatgagtactaagtgttgggg        796
Neisseria       caaacaggattagataccctggtagtccacgccctaaacgatgtcaattagctgttgggc        769
Klebsiella      caaacaggattagataccctggtagtccacgccgtaaacgatgtcgatttggaggttgtg        830
Pseudomonas     caaacaggattagataccctggtagtccacgccgtaaacgatgtcgactagccgttggga        820
                **** ***************** ************ *********     *       *


Brucella        tgt-ttacacttcggtggcgcagctaacgcattaaacattccgcctggggagtacggtcg        821
Rickettsia      gat--tctctttcggtttcgcagctaacgcattaagcactccgcctggggagtacggtcg        836
Mycoplasma      taactca-------gcgctgcagctaacgcattaagtactccgcctgagtagtatgctcg        849
Neisseria       aacctgattgcttggtagcgtagctaacgcgtgaaattgaccgcctggggagtacggtcg        829
Klebsiella      cccttgaggcgtggcttccggagctaacgcgttaaatcgaccgcctggggagtacggccg        890
Pseudomonas     tccttgagatcttagtggcgcagctaacgcgataagtcgaccgcctggggagtacggccg        880
                              * *********   **     ******* * **** *  **


Brucella        caagattaaaactcaaaggaattgacgggggcccgcacaagcggtggagcatgtggttta        881
Rickettsia      caagattaaaactcaaaggaattgacgggggctcgcacaagcggtggagcatgcggttta        896
Mycoplasma      caagagtgaaactcaaaggaattgacggggacccgcacaagtggtggagcatgtggttta        909
Neisseria       caagattaaaactcaaaggaattgacggggacccgcacaagcggtggatgatgtggatta        889
Klebsiella      caaggttaaaactcaaatgaattgacgggggcccgcacaagcggtggagcatgtggttta        950
Pseudomonas     caaggttaaaactcaaatgaattgacgggggcccgcacaagcggtggagcatgtggttta        940
                ****  * ********* ************ * ******** ******  *** ** ***


Brucella        attcgaagcaacgcgcagaaccttaccagcccttgacatcccggtcgcggttagtggaga        941
Rickettsia      attcgatgttacgcgaaaaaccttaccaacccttgacatggtggttatggattgcagaga        956
Mycoplasma      attcgaagcaacacgaagaaccttaccagggcttgacatccagtgcaaagctatag----        965
Neisseria       attcgatgcaacgcgaagaaccttacctggtcttgacatgtatggaatcctccagagacg        949
Klebsiella      attcgatgcaacgcgaagaaccttacctggtcttgacatccacagaactttccagagatg        1010
Pseudomonas     attcgaagcaacgcgaagaaccttacctggccttgacatgctgagaactttccagagatg        1000
                ****** *  ** ** * *********    ********


Brucella        cactatccttcagttaggctggaccggagacaggtgctgcatggctgtcgtcagctcgtg        1001
Rickettsia      tgctttccttcagttcggctgggccacacacaggtgttgcatggctgtcgtcagctcgtg        1016
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Mycoplasma      aga----tatagtagaggttaacattgagacaggtggtgcatggttgtcgtcagttcgtg        1021
Neisseria       gag----gagtgccttcgggagccgtaacacaggtgctgcatggctgtcgtcagctcgtg        1005
Klebsiella      gat----tggtgccttcgggaactgtgagacaggtgctgcatggctgtcgtcagctcgtg        1066
Pseudomonas     gat----tggtgccttcgggaactcagacacaggtgctgcatggctgtcgtcagctcgtg        1056
                           *         * ******* ******* ********* *****


Brucella        tcgtgagatgttgggttaagtcccgcaacgagcgcaaccctcgcccttagttgccagcat        1061
Rickettsia      tcgtgagatgttgggttaagtcccgcaacgagcgcaacccttattcttatttgccagcgg        1076
Mycoplasma      ccgtgaggtgttgggttaagtcccgcaacgaacgcaacccttgtcgttagttactaacat        1081
Neisseria       tcgtgagatgttgggttaagtcccgcaacgagcgcaacccttgtcattagttgccatcat        1065
Klebsiella      ttgtgaaatgttgggttaagtcccgcaacgagcgcaacccttatcctttgttgccagcgg        1126
Pseudomonas     tcgtgagatgttgggttaagtcccgtaacgagcgcaacccttgtccttagttaccagcac        1116
                  ****  ***************** ***** *********     **  ** * * *

Brucella        tcagttgggca--ctctaaggggactgccggtgataagccgagaggaaggtggggatgac        1119
Rickettsia      gtaatgccgggaactataagaaaactgccggtgat-aagccggaggaaggtggggacgac        1135
Mycoplasma      -taagttgagaa-ctctaacgagactgctag-tgt-aagctagaggaaggtggggatgac        1137
Neisseria       -tcagttgggca-ctctaatgagactgccggtgac-aagccggaggaaggtggggatgac        1122
Klebsiella      ttcggccgggaa-ctcaaaggagactgccagtgat-aaactggaggaaggtggggatgac        1184
Pseudomonas     ctcgggtgggca-ctctaaggagactgccggtgac-aaaccggaggaaggtggggatgac        1174
                         **  **    *****  *     *  *  ************** ***

Brucella        gtcaagtcctcatggcccttacgggctgggctacacacgtgctacaatggtggtgacagt        1179
Rickettsia      gtcaagtcatcatggcccttacgggttgggctacacgcgtgctacaatggtgtttacaga        1195
Mycoplasma      gtcaaatcatcatgccccttatgtcctgggctacacacgtgctacaatggctggtacaaa        1197
Neisseria       gtcaagtcctcatggcccttatgaccagggcttcacacgtcatacaatggtcggtacaga        1182
Klebsiella      gtcaagtcatcatggcccttacgaccagggctacacacgtgctacaatggcatatacaaa        1244
Pseudomonas     gtcaagtcatcatggcccttacggccagggctacacacgtgctacaatggtcggtacaaa        1234
                ***** ** ***** ****** *    ***** *** ***  ********     ***

Brucella        gggcagcgagcacgcgagtgtgagctaatctcc-aaaagccatctcagttcggattgcac        1238
Rickettsia      gggaagcaagacggtgacgtggagcaaatccct-aaaagacatctcagttcggattgttc        1254
Mycoplasma      gagttgcaatcctgtgaaggggagctaatctcaaaaaaccagtctcagttcggattgaag        1257
Neisseria       gggtagccaagccgcgaggcggagccaatctcacaaaaccgatcgtagtccggattgcac        1242
Klebsiella      gagaagcgacctcgcgagagcaagcggacctcataaagtatgtcgtagtccggattggag        1304
Pseudomonas     gggttgccaagccgcgaggtggagctaatcccataaaaccgatcgtagtccggatcgcag        1294
                * *  ** *    * **     ***  * * *  ***     **  *** ***** *

Brucella        tctgcaactcgagtgcatgaagttggaatcgctagtaatcgcggatcagc-atgccgcgg        1297
Rickettsia      tctgcaactcgagagcatgaagttggaatcgctagtaatcgcggatcagc-atgccgcgg        1313
Mycoplasma      tctgcaactcgacttcatgaagccggaatcactagtaatcgcgaatcagctatgtcgcgg        1317
Neisseria       tctgcaactcgagtgcatgaagtcggaatcgctagtaatcgcaggtcagc-atactgcgg        1301
Klebsiella      tctgcaactcgactccatgaagtcggaatcgctagtaatcgtagatcaga-atgctacgg        1363
Pseudomonas     tctgcaactcgactgcgtgaagtcggaatcgctagtaatcgtgaatcaga-atgtcacgg        1353
                ************   * *****  ****** **********    ****  **    ***

Brucella        tgaatacgttcccgggccttgtacacaccgcccgtcacaccatgggagttggttttaccc        1357
Rickettsia      tgaatacgttctcgggccttgtacacactgcccgtcacgccatgggagttggttttacct        1373
Mycoplasma      tgaatacgttctcgggtcttgtacacaccgcccgtcacaccatgagagttggtaatacca        1377
Neisseria       tgaatacgttcccgggtcttgtacacaccgcccgtcacaccatgggagtggggg------        1355
Klebsiella      tgaatacgttcccgggccttgtacacaccgcccgtcacaccatgggagtgggttgcaa--        1421
Pseudomonas     tgaatacgttcccgggccttgtacacaccgcccgtcacaccatgggagtgggttgctcca        1413
                *********** **** *********** ********* ***** **** **

Brucella        gaaggcgctgtgctaaccgcaaggaggcaggcgaccacggtagggtcagcgactggggtg        1417
Rickettsia      gaaggtggtgagctaacgcaag--aggcagccaaccacggtaaaattagcgactggggtg        1431
Mycoplasma      gaagtaggtagcttaaccgtttggagagcgcttcccaaggtaggactagcgattggggtg        1437
Neisseria       ------------------------------------------------------------        1355
Klebsiella      ------------------------------------------------------------        1421
Pseudomonas     gaagtagctagtctaaccgcaagggggacggttaccacggagtgattcatgactggggtg        1473


Brucella        aagtcgtaacaag-------------------------------------        1430
Rickettsia      aagtcgta-----------------------------------------        1439
Mycoplasma      aagtcgtaacaaggtatccgtacgggaac---------------------        1466
Neisseria       ------------------------------------------------        1355
Klebsiella      ------------------------------------------------        1421
Pseudomonas     aagtcgtaacaaggtagccgtaggggaacctgcggctggatcacctccttt       1524
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