DETECTION OF SOME MULTILOCUS GENES AMONG *BRUCELLA MELITENSI*S ISOLATED FROM PATIENTS SUFFERING FROM BRUCELLOSIS

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ABSTRACT:

In this study, 350 blood samples were collected from patients suffering from clinical signs and symptoms with brucellosis, these samples were raveled that 350 were IgM-IgG cassette positive patients consist of 117(33.42%) male and 233(66.57%) females. The samples were cultured on different types of media to identified *Brucella* spp. The results showed that, out of 350 IgM-IgG cassette positive, only 33(9.42%) samples were growth on specific *Brucella* medium, while 317(90.57%) samples were growth on other types of media revealed to other types of bacteria, the identification of *Brucella melitensis* by direct DNA extraction from blood samples was investigated, it was found that out of 350 blood culture samples, only 4(1.14%) isolates were related to *Brucella melitensis* positive at (223bp). In addition, *Brucella melitensis* isolation was identified by Vitek 2 system, and then subjected to PCR, out of 22 isolates, 16(72.7%) gave positive results for the identification of *Brucella* in clinical samples when primer pair is used. The primer pair used in this study succeeded in the amplification of a (223bp) fragment from *Brucella* spp. isolates culture that were studied. Multilocus genes of *Brucella melitensis* were detected in 20 *Brucella melitensis* isolates, it was found that, all multilocus genes was found in these isolates, the gap gene was found at (589bp) bands when compared with allelic ladder, aroA gene was found at (565bp) bands when compared with allelic ladder, glk was found in at (475bp) bands when compared with allelic ladder, dnak gene was found at (470bp) bands when compared with allelic ladder.

**Keywords:** Multilocus Genes, *Brucella melitensis*, Brucellosis, PCR.

I. INTRODUCTION:

Brucellosis is a zoonotic disease affecting animals and human in many parts of the world, it is required eradication of infection that prolonged treatment (Franc et al, 2018). Animals are considered as natural reservoir of human brucellosis genetic and immunological evidence indicates that all members of the *Brucella* genus are closely related, nevertheless, based on relevant differences in host preference and epidemiology displayed by the major variants, as well as molecular evidence of genomic variation, it has many virulence factors causing severe Pathogenicity (Moreno, 2020). Chronic infection may result in infection of secondary tissues and may lead to endocarditis, osteoarthritis, spondylitis, arthritis, osteomyelitis, meningitis, and severe neurological disorders. Symptoms may also recur years after the original infection (Doganay et al, 2019). The World Health Organization (WHO) laboratory biosafety manual classifies *Brucella* in risk group III. Brucellosis is one of the five bacterial zoonosis, worldwide distribution, caused by organism belonging to genus *Brucella*, but rarely leading to death (Solanki). Brucellosis is worldwide distribution and economically the most ravaging that is associated with chronic serious sequels in humans (Solanki and Devi, 2020). Brucellosis is the most common zoonotic infection worldwide with more than 500,000 people diagnosed each year, the global disease burden is also immense. Disease is endemic in the Middle East, the Balkan Peninsula, Central Asia, and regions of Africa and Latin America (Hull and Schumaker, 2018). The risk of disease and its severity is to a significant extent determined by the type of *Brucella* to which an individual is exposed. This will be influenced by the species of host animal acting as source of infection (Mabbott, 2018). *B. melitensis* is the type most frequently reported as a cause of human disease and the most frequently isolated from cases. It is the most virulent type and associated with severe acute disease. It is recorded as endemic in several countries and accounts for a disproportionate amount of human brucellosis (Rouzic et al,
2020). The organism is normally associated with infection in sheep and goats, but other species, including dogs, cattle and camels can be infected. In some countries, particularly in the Middle East, *B. melitensis* infection of cattle has emerged as an important problem. Contrary to some traditional views, *B. melitensis* remains fully virulent for man after infecting cattle (Pal et al., 2017). Multilocus sequencing typing (MLST), as a genotyping tool for assessing genetic diversity and relationships, was widely used to identify and analyze diversity of bacteria and epidemiology characterization (Rasoamanana et al, 2020). A multilocus genotype is the combination of alleles found at two or more loci in a single individual. For example, in a diploid species, if there are two SNP loci and the first locus has alleles A and G, while the second locus has alleles T and C, the multilocus genotype can be represented as {A/G,T/C} (Yonemitsu et al., 2019). If the genome is not haploid then the multilocus genotype does not necessarily determine which alleles co-occur on chromosomes. In the example, if the two loci are located on the same chromosome the possibilities are either {A-T, G-C} or {A-C,G-T} (Randel, 2017).

Aim of the Study:

The study aimed to detect the *Brucella* spp. isolates in patients with brucellosis and to study of some multilocus genes.

II. MATERIAL AND METHODS:

350 patients were obtained from routine history and clinical signs. Information was arranged in a questionnaire including: name, age, sex, disease duration, animal products consumption. A total of 350 blood samples were collected from patients suffering from clinical signs and symptoms with Brucellosis. There were positive of IgM-IgG cassette positive, clinical signs, history of epidemiological exposure. They were admitted to many hospitals: Babylon Hospital for Maternal and Pediatrics, Al-Hilla Surgical Teaching Hospital, Al- Qassem General Hospital, Al- Hashimia General Hospital, Marjan Hospital, Central Health laboratory, and private laboratories, during the period from January-2020 to February, 2021.

Ethical approval:

All subjects involved in this work are informed and the agreement will obtained verbally from each one before the collection of samples. This study was approved by the committee on publication ethics at College of medicine, Al-Qadisiyah University, Iraq.

Colonial morphology and microscopic examination:

A single colony from each primary positive culture on blood and nutrient agar and identify it depending on its morphological properties (colony shape, size, color, borders, and texture) and exam it by light microscope after being stained with Gram’s stain. After examination it, biochemical tests were done on each isolates to complete the finale identification (Baron et al, 1994; Collee et al, 2006 and MacFadden, 2000).

Growth characteristics:

*Brucella* colonies generally become visible after the cultures have been incubated for 3 days, but if there is no growth the plates should be routinely examined on the fourth or fifth up to 3 week especially when selective media is used (Alton et al, 1988). Which the growth colony has *Brucella* colony appearance which are translucent and of pale honey color and when viewed from above, the colony appeared convex and pearly white. It would be submitted to other biochemical tests. If there is any contamination, isolated *Brucella* colony were picked up and re-streaked on selective medium (trypticase soy agar with added antibiotic mixture, *Brucella* agar, blood agar and Chocolate agar) after cultivation on broth media.

Tentative identification by slide agglutination:

A slide was placed in Petri dish, then a small portion of growth picked up by a sterile loop and emulsified in 3 drops of saline. A drop of anti *Brucella* serum (A, M) was added then mixed and examined for agglutination as described by (Alton et al, 1988).

Gram's stain and Modified Ziehl- Neelsen method:

Gram stain as described by Quinn et al, (2004). While modified Ziehl- Neelsen method is used as described by Alton et al., (1988). temporarily, after the smear was dried and fixed it was stained with diluted carbol fuchsin for 10 min. washed before differentiate with 0.5% acetic acid for 30 sec., washed again then counterstained with 1%
methylene blue solution for 20 sec. It is used especially for tentative *Brucella* identification from blood samples. *Brucella* organisms should stain red against a blue background.

**Definitive Identification by VITEK 2 – Compact:**

The identification of *Brucella* was confirmed using VITEK 2-Compact which represents an advanced colorimetric technology for bacterial identification, gram negative (GN) card was used for this purpose for *Brucella* Identification.

**Procedure:**

All the following steps were done according to the manufacturer’s instructions (Biomerieux, France).

1. Three ml of normal saline was placed in a plane test tube and inoculated with a loop full of isolated colony

2. The test tube was inserted into the Dens Check machine for standardization of the colony to McFarland standard solution (1.5X10\(^8\) cfu/ml).

3. The standardized inoculum was placed into the cassette and a sample identification number entered into the computer software via barcode.

4. The VITEK-2 card type was then read from barcode placed on the card during manufacture and the card was thus connected to the sample identification port.

5. The cassette was placed in the filter module, when the card were filled, transferred the cassette to the reader/incubator module.

All the subsequent steps were handled by the instruments; the instrument controls the incubation temperature, the optical reading of the cards and continuously monitors and transfers test data to the computer for analysis. When the test cycle was completed, the system automatically ejected the cards into a waste container.

**DNA Extraction and purification:**

a. The main basic approach used for DNA purification was the one advised by Promega (the company that provided the Kit):

b. *Brucella* cells are harvested from the *Brucella* agar with PBS PH=6.4.

c. One ml of harvests cells were putted in to 1.5ml micro centrifuge tube and pelleted by centrifugation at 15,000 xg for 3 min.

d. The cells were washed once with PBS and twice with double distilled water by centrifugation at 4000xg for 5 min.

e. 200μl of the cellular pellets placed in sterile micro centrifuge tube treated by the addition of 600μl of nuclease solution and incubated at 80 °C for 5 min (to lyses the cells).

f. Then cooled to room temperature before the addition of 200μl of protein precipitation solution which then vortexed vigorously at high speed for 20 seconds and chilled on ice for 5 minutes, then centrifugation for 10 minutes at 13,000 xg.

g. The supernatant containing the DNA was transferred to a clean 1.5ml micro centrifuge tube containing 600μl of room temperature isopropanol then it was mixed by inversion until the white thread-like strands of DNA form a visible mass.

h. The tube was centrifuged at 13,000 xg for 1 min, then supernatant was decanted.
The DNA pellet washed in 600 μl of 70% ethanol and centrifuged as above. The tube drained on a clean absorbent paper and allowed the pellet to air-dry for 15 min. rehydrated by adding 30μl of TE (rehydration solution) than stored at -20°C.

**Preparation of primers suspension of multilocus genes:**

The primers were suspended by dissolving the lyophilized primers after spinning down with TE buffer depending on manufacturer instruction as stock suspension. Working primer tube was prepared by diluted with TE buffer. The final picomoles depended on the procedure of each primer.

**Primers Sequences:**

The primers sequences and PCR conditions that used in study are listed in Table (1) (Gopaul *et al.*, 2008).

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequence (5’-3’)</th>
<th>Size of bp</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>16Sr RNA</td>
<td>F: 5’TGGCTCGGTTGCAATATCAA-3’&lt;br&gt;R: 5’-CGCGCTTGCTTTCCAGTGCTG-3’</td>
<td>223</td>
<td>Step 1: 95ºC, 5 min.&lt;br&gt;Step 2: 95ºC, 35 sec.&lt;br&gt;Step 3: 50ºC, 30 sec.&lt;br&gt;Step 4: 72ºC, 40 sec.&lt;br&gt;Step 5: 72ºC, 5 min.&lt;br&gt;Step 7: 4ºC, forever</td>
</tr>
<tr>
<td>gap</td>
<td>F: 5’ YGCAAAGCAGGTCATCGT 3’&lt;br&gt;R: 5’ GCGGYTGAGAAGCCCA 3’</td>
<td>589</td>
<td>Step 1: 95ºC, 5 min.&lt;br&gt;Step 2: 95ºC, 35 sec.&lt;br&gt;Step 3: 50ºC, 30 sec.&lt;br&gt;Step 4: 72ºC, 40 sec.&lt;br&gt;Step 5: 72ºC, 5 min.&lt;br&gt;Step 7: 4ºC, forever</td>
</tr>
<tr>
<td>aroA</td>
<td>F: 5’ GACCAATCGACGTGGGGG 3’&lt;br&gt;R: 5’ YCATCAKGGGCATGAATTC 3’</td>
<td>565</td>
<td>Step 1: 95ºC, 5 min.&lt;br&gt;Step 2: 95ºC, 35 sec.&lt;br&gt;Step 3: 50ºC, 30 sec.&lt;br&gt;Step 4: 72ºC, 40 sec.&lt;br&gt;Step 5: 72ºC, 5 min.&lt;br&gt;Step 7: 4ºC, forever</td>
</tr>
<tr>
<td>glk</td>
<td>F: 5’ TATGGAAMAGATCGGCGG 3’&lt;br&gt;R: 5’ GGGCCTTTGTCTCGAAGG 3’</td>
<td>475</td>
<td>Step 1: 95ºC, 5 min.&lt;br&gt;Step 2: 95ºC, 35 sec.</td>
</tr>
</tbody>
</table>
III. RESULTS AND DISCUSSION:

Samples collection of Human patients:
In this study, 350 blood samples were collected from patients suffering from clinical signs and symptoms with brucellosis, these samples were revealed that 350 were IgM-IgG cassette positive patients consist of 117 (33.42%) male and 233 (66.57%) females, the results were shown in Table (2), Figure (1) These samples were taken under supervision of consultant doctors, the clinical signs, were chills, and sweats, aches, lack of energy, joint and back pain, arthritis, spinal tenderness, headache, loss of appetite, weight loss, constipation, abdominal pain, diarrhea, cough, testicular pain/epididymo orchitis, rash, splenomegaly and hepatomegaly. The most common serological tests utilized in both livestock and humans are the serum agglutination test (SAT), the complement fixation test (CFT), buffered Brucella antigen tests including the IgM-IgG cassette and the U.S. Card test, ELISA, and the fluorescence polarization assay (FPA) (Ducrotoy et al., 2018; Saavedra et al., 2019).

Table (2): IgM-IgG cassette positive of Human patient's samples

<table>
<thead>
<tr>
<th>No. of IgM-IgG cassette positive</th>
<th>males</th>
<th>%</th>
<th>females</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>350 samples</td>
<td>117</td>
<td>33.42</td>
<td>233</td>
<td>66.57</td>
</tr>
</tbody>
</table>

These results were agreement with results obtained by (Kosack et al., 2018; Jansen et al., 2019 and Deng et al., 2019) who found that, the diagnosis of human brucellosis cannot be made solely on clinical grounds due to the wide variety of clinical manifestations of this disease, and it is essential to perform bacteriological and serological
tests. However, all physicians dealing with a febrile patient living in an endemic area or recently travelled to a country where brucellosis is endemic (“travel-associated disease”) must be aware of the possibility that the patient could be infected with Brucella (Zange et al., 2019). For this reason, correct clinical history taking is essential to orientate the diagnosis, and the need for some very basic questions (profession, food ingested, contact with animals and travel to endemic areas) must be emphasized. Moreover, a rapid screening test must be performed (Pal et al., 2017; Gusi et al., 2019). Should the screening test prove negative in the face of a history and clinical presentation, it is advisable to check the result using additional tests. Careful observance of these practices will help to avoid delayed diagnosis (Karimi & Mosavari, 2019).

Identification of Brucella spp. by biochemical test:
The samples were cultivated on different types of media at 37°C for (1-3 weeks). The identification of Brucella was depended on colony morphology, microscopically, and biochemical tests as initial identification. Brucella colonies were coccobacilli, Gram-negative, aerobic, non-spore-forming, non-motile and non-capsulated (Kosoy et al., 2019). Although able to multiply in life-less media, Brucella organisms are better described as facultative extracellular intracellular parasites. The Brucella are members of the α–proteobacteria, and interestingly (being animal pathogens) have close relationships with soil organisms, with plant symbionts, and with phytopathogens (Machelart et al., 2020). In this study, the samples were cultured on different types of media to identified Brucella spp. the results showed that, out of 350 rose bengal positive, only 33(9.42%) samples were growth on specific Brucella medium, while 317(90.57%) samples were growth on other types of media revealed to other types of bacteria. The results were shown in Table (3).

Table (3): Identification of Brucella spp. according to growth on specific media

<table>
<thead>
<tr>
<th>No. of IgM-IgG cassette Test positive</th>
<th>growth on specific Brucella medium</th>
<th>%</th>
<th>Other types of bacteria</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>350 samples</td>
<td>33</td>
<td>9.42</td>
<td>317</td>
<td>90.57</td>
</tr>
</tbody>
</table>

Brucella species grow slowly on primary cultures and subcultures, while their inert biochemical profiles hamper fast identification of isolates; however, the sensitivity of this technique is low, ranging from 15 to 70% (Yagupsky et al., 2019). Consequently, detection and identification of Brucella spp. in clinical specimens by cultures may still be a difficult task with significant delays and hazards to lab personal. Brucella spp. are class III pathogens, since their handling poses considerable risk to laboratory personal (Bazzi et al., 2017). The identification of Brucella was depended on colony morphology, microscopically, and biochemical tests as initial identification as shown in Table (4).

Table (4): Differential biochemical tests for Brucella spp. of human isolates

<table>
<thead>
<tr>
<th>No.</th>
<th>Biochemical test</th>
<th>Brucella spp. result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Brucella melitensis</em> antigen test</td>
<td>+ve</td>
</tr>
<tr>
<td>2.</td>
<td>Indol test, Lactose</td>
<td>-ve</td>
</tr>
<tr>
<td>3.</td>
<td>MRVP</td>
<td>-ve</td>
</tr>
<tr>
<td>4.</td>
<td>Hemolysin, Gelatin</td>
<td>-ve</td>
</tr>
<tr>
<td>5.</td>
<td>Litmus test, Citrate test</td>
<td>-ve</td>
</tr>
<tr>
<td>6.</td>
<td>Motility test</td>
<td>-ve</td>
</tr>
<tr>
<td>7.</td>
<td>Nitrate test</td>
<td>+ve</td>
</tr>
<tr>
<td>8.</td>
<td>Catalase, Oxidase, Urease</td>
<td>+ve</td>
</tr>
<tr>
<td>9.</td>
<td>H₂S</td>
<td>-ve</td>
</tr>
</tbody>
</table>

Blood samples have traditionally been cultured using biphasic medium such as Castaneda medium or various subculture techniques. However, these methods detect Brucella species in only 40-90% of acute human cases and 5-20% of chronic or focal cases. Diagnosis may also be substantially delayed as occasionally incubation times as long as 6 weeks are necessary (Castaño-Zubieta et al., 2021). For human samples, automated culture systems have largely replaced these traditional methods (Yu et al., 2018). The result has been higher levels of detection and shorter incubation 58 times. The duration of bacteremia in patients is poorly characterized; however, once focal
infection develops fewer brucellae are present in the blood resulting in decreased sensitivity of blood culture in patients with chronic brucellosis (Yagupsky et al., 2019).

**Identification of Brucella melitensis by direct DNA extraction and 16Sr PCR based assay:**

In this study, the identification of *Brucella melitensis* by direct DNA extraction from blood samples was investigated, it was found that out of 350 blood culture samples, only 4(1.14%) isolates were related to *Brucella melitensis* positive at (223bp) as shown in Table (5). In addition, *Brucella melitensis* isolation was identified by Vitek 2 system, and then subjected to PCR, out of 22 isolates, 16(72.7%) gave positive results for the identification of *Brucella* in clinical samples when primer pair is used. The primer pair used in this study succeeded in the amplification of a (223bp) fragment from *Brucella* spp. isolates culture that were studied. This means that the DNA extracted from culture harboring *Brucella*, these results were identical with Kaden et al., (2017) which had confirmed detection of *Brucella* DNA in clinical specimen by PCR assay. All *Brucella* isolates that were studied with PCR have the same 223bp fragment as shown in Table (5) and Figure (2).

**Table (5) Identification of Brucella melitensis by direct DNA extraction and 16SrPCR gene based assay:**

<table>
<thead>
<tr>
<th>Blood samples</th>
<th>On blood culture</th>
<th>Vitek2 system</th>
<th>Direct DNA extraction</th>
<th>16Sr RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>350</td>
<td>33(9.42%)</td>
<td>22(66.6%)</td>
<td>4(1.14%)</td>
<td>16(72.7%)</td>
</tr>
<tr>
<td>Total B. melitensis</td>
<td></td>
<td></td>
<td></td>
<td>20 isolates</td>
</tr>
</tbody>
</table>

Figure (2): agarose gel electrophoresis (1.5%) of RCR amplified of 16SrRNA gene (223 bp) of *Brucella melitensis* for (55) min at (70) volt L: ladder (DNA marker), (1, 2, 3, 4, 5, 6, 7, 8, to 16) Amplify of 16Sr RNA gene in clinical isolates of *Brucella melitensis*.

A study of Dal et al., (2019) who found that, the PCR amplification procedure with those of routine blood culture for the diagnosis of 30 cases of patients suspected of brucellosis culture positive at rate 73.4%, while Açıkgöz et al., (2018) found that PCR positive of *Brucella* in rate 93.3%. Results of PCR were the same as those obtained by Paul et al., (2020) who used PCR amplification contained a single pair of oligonucleotide primers designed to amplify a 223 bp product and demonstrated that the assay was sensitive and specific for *B. melitensis*. In the present experiment, the primers for B4 and B5, described by El-Diasty et al., (2018), were used for molecular detection of *B. melitensis*. The authors compared these primers with the nucleotide sequence databases Genbank and EMBL (European Molecular Biology Laboratory) and did not identify any significant sequences homology with other bacteria. These primers have also been used with success to diagnose infection with *Brucella* by various authors (Lecchi et al, 2019; Mol et al, 2020). None of whom ever related false positives. Kaden et al., (2017) and Dadar et al., (2019) have found that PCR-based assays have been explored for the rapid detection and confirmation of *Brucella*, which in a new assay in the diagnosis of the most infective pathogen. Conventional Polymerase chain reaction-based, real-time PCR, multiplex PCR, mono plex PCR are used. These results agree with the present study.

**Detection of multilocus genes of Brucella melitensis:**

Many studies were already successfully determined the sequences of multiple genetic loci in order to examine the relationships between *Brucella* isolates (Sacchini et al, 2019), (Yespembetov et al, 2019), (Ashford et al, 2020). In this study, multilocus genes of *Brucella melitensis* were detected in 20 *Brucella melitensis* isolates, it was found that, all multilocus genes was found in these isolates, the gap gene was found at (589) bp bands when compared...
with allelic ladder as shown in Figure (3), aroA gene was found at (565) bp bands when compared with allelic ladder as shown in Figure (4), glk was found in at (475) bp bands when compared with allelic ladder as shown in Figure (5), dnak gene was found at (470) bp bands when compared with allelic ladder as shown in Figure (6).

Figure (3): agarose gel electrophoresis (1.5%) of RCR amplified of gap gene (589 bp) of Brucella melitensis for (55) min at (70) volt L: ladder (DNA marker), (1, 2, 3, 4, 5, 6, 7, 8, to 20) Amplify of gap gene in clinical isolates of Brucella melitensis.

Figure (4): agarose gel electrophoresis (1.5%) of RCR amplified of aroA gene (565 bp) of Brucella melitensis for (55) min at (70) volt L: ladder (DNA marker), (1, 2, 3, 4, 5, 6, 7, 8, to 20) Amplify of aroA gene in clinical isolates of Brucella melitensis.

Figure (5): agarose gel electrophoresis (1.5%) of RCR amplified of glk gene (475 bp) of Brucella melitensis for (55) min at (70) volt L: ladder (DNA marker), (1, 2, 3, 4, 5, 6, 7, 8, to 20) Amplify of glk gene in clinical isolates of Brucella melitensis.
IV. DISCUSSION:

A study of Pisarenko et al., 2018, (Zhao et al., 2020) who found that multilocus sequence typing trees in the study of Brucella roughly approximate the whole-genome phylogeny but use only four housekeeping genes. Barker, (2018) found that, nine discrete genomic loci corresponding of sequence were examined from Brucella isolates. Multilocus sequence typing (MLST) has become a useful tool for studying the genetic diversity of important public health pathogens. However, the lack of a sole standardized scheme represents the greatest limitation regarding typing this species (Patiño et al., 2018). The use of housekeeping genes was essential to the design of MLST; besides ensuring their presence, and thereby the typability of the strain, the slow evolution of these genes made MLST an appropriate tool for studying the long term evolution of the population structure of a species on a global scale (Blankenship et al., 2020). The use of multilocus sequence data has two particular advantages. Clearly, and of particular relevance in the case of a genetically conserved group such as Brucella, the additive use of multiple loci increases the discriminatory capacity compared to that that can be obtained when using a single target. Secondly, loci can be selected that are spaced far enough apart such that any pairs of alleles are unlikely to be inherited together by recombination (Vergnaud et al., 2018). This is important as recombination can distort the apparent relationships between similar isolates if they are characterized at only a single locus. Thus studies based on multilocus approaches that buffer against possible recombination are more desirable than the characterization of individual loci. It was recently suggested that such an approach should be applied by taxonomists to large samples of groups of closely-related bacteria, and especially to those where species delineation has historically been difficult, to determine whether genotypic clusters can be delineated, and to guide the definition of species (Liang et al., 2020). Detection and analysis of polymorphisms with RFLP is also used for identification and characterization of Brucella species with some advantages such as applicability, easy interpretation and could be used for numerous samples. It was used the of omp25 genes, to characterize all species of Brucella from each other and detect their biovars (Yang et al., 2020). Suárez-Esquivel et al., (2020) found the dnaK gene and surrounding sequences from reference strains of the Brucella species were amplified by the polymerase chain reaction (PCR) with primers chosen according to the published sequence of the B. melitensis dnaK gene and studied for polymorphism with nine restriction endonucleases. Multiple locus methods categorically eclipsed their single locus antecedents, excepting their occasional use as an additional enhancing locus. These multiple locus methods were only incrementally more difficult, but offered a much higher resolution alternative. In some cases, it was possible for single hyper variable genes to mutate faster than the actual spread of a pathogen. In an outbreak investigation, this could distort the apparent number of sources (Kimura et al., 2017). As their names imply, these new approaches increase the number of target loci from fewer than ten to hundreds or thousands. The key difference between these two systems is how faithfully they adhere to the original MLST concepts (Barker, 2018).

V. CONCLUSION:

B. melitensis considered the main zoonosis species of Brucella genus that cause serious disease for human. Multi-locus genes (gap, aroA, glk, dnaK, gyrB, trpE, omp25, cobQ, Int-hyp) were housekeeping genes consist of alleles on different loci on the total genome, it was considered important tool for detection, identification, genotyping and phylogenic.


