

Diagnostic Study on *Brucella melitensis* isolated from Human in Wassit province

Basil A. Abbas*, Haethem Q. Mohammed** and Jalal A. Tofah* **

*College of Veterinary Medicine, Basrah University.

**Department of Clinical Laboratory Sciences, College of Pharmacy, Al-Mustansiriyah University.

*** Al-karamah Teaching hospital, kut province

الخلاصة:

أجريت هذه الدراسة للتعرف على مدى الإصابة بمرض البروسيلا المالطية في الإنسان لمدينة الكوت للمدة ما بين شهر أيلول لعام 2007 ولغاية شهر مايس لعام 2008.

جمعت 100 عينة دم مشتبه بإصابتها بمرض البروسيلا (داء البروسيلات) و 20 عينة دم كانت غير مصابة بمرض البروسيلوسز وليس لها تاريخ الإصابة بالمرض وحسب نتائج الفحوص المصلية المجرة (الروزينكال و 2 - المركابتوايثانول). فقد بلغت حالات الإصابة بالنسبة للعينات المشتبه أصابتها بالمرض 58 حالة (58%).

بينت الدراسة وباستعمال فحص 2-المركابتوايثانول بان عدد العينات الموجبة للإصابة بالطور المزمن للمرض قد بلغت 12 عينة (20,68%).

استخدمت عينات دم من الحالات المصابة لغرض عزل البكتريا ثم استعملت بعدها طريقة العزل بواسطة كاستيندا ثنائي الطور، وجاءت النتائج بالحصول على 8 عزلة من مجموع 58 عينة بنسبة 20% وقد تميزت الطريقة بقصر المدة الزمنية للعزل.

أجريت عدد من الفحوص الشكلية والكيميوية لتشخيص العزلات الإحدى والأربعون، من ضمنها الفحص المجهرى المباشر وفحص الكاتليز والاكسيديز واليوريز وفحص IMViC والزراعة في وسط اختزال النترات والوسط الحاوي على الصبغات المثبطة للنمو البكتيري بوجود صبغة الفوكسين القاعدي وبتركيزين هما 1:50000 و 1:100000.

أظهرت نتائج دراسة تحديد النمط الحيوي للعزلات حيث ان (2) عزلتين من مجموع (8) عزلات بنسبة 19.51% قد تلازنت فقط مع المصل الأحادي الخاص لـ *Brucella abortus* (A) لذا كان نمطها الحيوي 2. بينما الـ 6 عزلات المتبقية بنسبة 75% قد تلازنت في آن واحد مع المصل الحادي الخاص بالـ *Brucella abortus* والمصل الأحادي الخاص بالـ *Brucella melitensis* (M) وكان نمطها الحيوي 3.

كما أظهرت الدراسة ان جميع العزلات المعزولة من الحالات المصابة كانت في طور الأملس وابدت جميع العزلات سالبيتها لاختبارات عوامل الضراوة متمثلة بإنزيمات (الهيمولايسين، الجيلاتينييز، اللستينيز، DNAase).

اجري فحص الحساسية لـ 22 مضاداً حيويّاً بطريقة الأقراص وأظهرت جميع العزلات حساسيتها لكل من Streptomycin, Piperacillin, Norfloxacin, Cephoxitin, Ciprofloxacin, Rifampicin and Doxycyclin بنسبة 100%. كانت نسبة كل من Chloramphenicol 87,5% وكل من Tetracycline و Gentamycin بنسبة 75% لكل منهما.

كانت مقاومة 100% لكل من Augmentin, Ceftriaxone, Amoxicillin, Nalidixic acid, Cloxacillin, lindamycin Cefixitime, Lincomycin, Trimethoprin و.

Abstract:

This study was performed to identify the extent of the infection with Brucellosis caused by *Brucella melitensis* in humans in Kut city through a period of nine months from September 2007 till May 2008.

One hundred blood samples were collected from humans and all were suspected in having brucellosis while twenty blood samples were collected from humans and all were known in not being suspected in having brucellosis and has no history in having such disease according to the performed serological tests (Rose Bengal test and 2-Mercaptoethanol test). The recorded cases for the brucellosis suspected samples were 58 Cases (58%) that positive for Rose Bengal test which the acute phases.

With the use of 2-Mercaptoethanol test, the results showed that the chronic phase positive cases reached up to 12 samples (20.68%).

Blood sample from the infected cases used for the purpose of bacterial isolation. Later by using Castaneda biphasic medium the results showed that out of 58, 8 (13.79%) isolates were positive for the test. This method has been characterized by its time shortness for isolation.

A number of morphological and biochemical tests had been carried including the direct examination, Catalase, Oxidase, Urease, IMVIC test, inoculation into nitrate reduction test medium and the ability to grow in the bacteriostatic dyes medium under the presence of basic fuchsin dye with two concentrations 1:50000 and 1:100000.

The results of the biotyping of isolates showed that 2 isolates out of 8 isolates (25%) were agglutinated with monospecific antisera for *Brucella abortus* (A) so it was belonging to the biotype 2. While the rest 6 isolates (75%) which were agglutinated simultaneously with monospecific antisera for *Brucella abortus* (A) and with monospecific antisera for *Brucella melitensis* (M) so it was belonging to the biotype 3.

All isolate's colonies which were isolated from the infected cases were in the smooth phase and showed its negativity to the virulence factors tests represented by the enzymes of (hemolysin, gelatin hydrolysis, lecithinase, DNAase).

All isolates were subjected to the antibiotics sensitivity test toward twenty two antibiotics and they were sensitive in a rate of 100% toward Streptomycin, Pipracillin, Norfloxacin, Ciprofloxacin, Rifampicin, Doxycyclin and Cephoitin while toward Chloramphenicol it was 92% and for Tetracycline and Gentamycin it was 80% for both. At the same time the isolates were resistant at a rate 100% to Augmentin, Ceftriaxone, Amoxicillin, Nalidixic acid, Lincomycin, Cefixine, Cloxacillin, Clindamycin, and the mixed Trimethoprin and Sulfamethoxazole.

Introduction:

Brucellosis is a contagious disease caused by bacteria of the genus *Brucella*. It is primarily a disease of humans and domestic animals mainly goats, sheep, cattle, pigs, dogs and camels and horses^[1].

The disease is named after David Bruce, a British army medical doctor, who isolated *Brucella melitensis* from the spleen of a dead British soldier on the island of Malta in 1887^[2].

In 1897 Danish veterinarian Bernhard Bung isolated *Brucella abortus* as the agent and the additional name Bang's disease was assigned. In modern usage Bung's disease is often shortened to just Bang's when ranchers discuss the disease or vaccine^[3]. The popular name (undulant fever) originates from the characteristic undulance (or wave - like nature) of fever which rises and falls over weeks in untreated patients In the 20th century, this name, along with Brucellosis after *Brucella* named from Dr. Bruce gradually replaced the 19th century names Mediterranean fever and Malta fever^[4].

Material and Methods:

Samples Collection: Humans Blood Samples Collection:

A total of 100 patients who attended Al – Zahra'a hospital and Al – Karama'a hospital in Kut city as well as 20 blood samples collected from humans without infected by brucellosis and according to the carrying out serums examining results (Rose Bengal Test and 2– Mercaptoethanol Test), during the period from September 2007 until May 2008. The patients were both sexes (males 57 and females 43). Patient's aged between 1.5 and 61 years. They were suffering from clinical signs such as fever, headache, sweating, joint pains and back pain.

Treated the Blood Samples:

According to clinical signs, from each patient was drawn 8–10 ml of venous blood for serological diagnosis and culture. Blood sample was collected in sterile 10 ml test tube was contained EDTA as anticoagulation material. 3 ml of each patient's

blood sample was put in sterile plain tube and centrifuged for 15 min 3000 rpm then the serum was made serological diagnosis. The serological tests were made as described by ^[5].

Rose Bengal Test (RBT):

It was used by specially Rose Bengal kit that kit was composed of *Brucella abortus* S99 killed by heating and 5% of phenol as well as treated by Tris – HCl, pH 3.62 and stained by Rose Bengal dye ^[6].

Two–Mercaptoethanol Test (2–ME)

It was carried out Rose Bengal test positive it like as completed test, special kit was used for this purpose and it was composed of two reagents are R1 and R2.

Procedure:

- A** - Fifty microliters of reagent 1 were added to eppendorf tube that give tube number 1 and same amount of reagent 2 were put in eppendorf tube number 2.
- B** - Fifty microliters of serum infect were drawn then added for each tube separately and incubated at 37 C° for 30 min.
- C** - Thirty microliters of each eppendorf tube were drawn and it put separately on sterile glass plate.
- D** - Taking thirty microliters of Rose Bengal kit and put near material on glass plate in above step then gently mixing of each alone by using plastic rod. After 4 min the result was read as following:

Reagent 1	Reagent 2	Results
Negative	Negative	Not agglutination: refers to absence of immunoglobulin (Ig) class G or presence in small amount and enable discovered by Rose Bengal test.
Positive*	Negative	Agglutination (R1) only: refers to presence IgM residues especially after case recovery.
Positive*	Positive*	Agglutination both (R1) and (R2): refers to presence IgM or/and presence IgG that indicated the infected countious with <i>Brucella</i> .
Positive*	Negative	Agglutination (R2) only: refers to a false result and the test should be return.

*** Result was refereed positive until that agglutination in small or fine.**

Note: Serum positive of patient or/and animals were preserved in the eppendorf tube and stored at –20 C° (Deep freezer) after the addition of sodium azaid (0.02%) as preservation material, until the use of ELISA Test.

Bacterial Isolation and Identification:

According to serological tests result mentioned previously collection of 4–5 ml of patient blood who gave positive result in one or both above tests was repeated: Under sterilized condition 4–5 ml of blood were injected directly in Castaneda biphasic medium then incubated at 37 C°. The plates were tested after 3 days of

incubation and the bacterial growth on solid medium was noted. At the same time 2– 3 drops of liquid medium were drawn by sterilized syringe which in turn used to vaccinate or inoculate selective medium *Brucella* agar supplemented with antibiotics. Then the plates were incubated to note the appearance of any bacterial growth and the plates were incubated again in case of absence of bacterial growth and tested once again after every 3 days and so on for 6–8 weeks.

When the bacterial growth appeared on plates surface the typical colonies were selected according to their morphological characteristics (pinpoint, smooth, translucent and glistening) to conduct diagnostic the isolates were further diagnosed by diagnostic biochemical and serological tests ^[7].

Diagnosis of Bacterial Isolates: Direct Microscopic Tests:

1- Gram's Stain:

According to Preston and Morrell (1962) method the Gram's stain solutions was used.

2- Modified Ziehle–Neelsen Stain:

Brucella appearance stained with red color and in shape of short bacilli to cococi while other types of bacterial stained blue ^[6].

Biochemical Tests:

- 1- Catalase Test
- 2- Oxidase Test
- 3- Urease Test
- 4- Indole Production Test
- 5- Methyl Red Test
- 6- Voges – Proskauer Test
- 7- Citrate Utilization Test
- 8- Nitrate Reduction Test
- 9- Lactose Fermentation Test
- 10- Hydrogen Sulfide (H₂S) Production Test

Bacterial Growth on Bacteriostatic Dye:

Stock solution of basic fuchsin dye was added to trypticase soy agar after cooling to about 55 C° then dye was mixed with agar and poured into Petri dishes. The plates were kept at 4 C° until used within 24 hrs suspension of recently growing *Brucella* cultures (loopful in 10 ml of sterile normal saline) were inoculated on dye plate by immersing a sterile cotton in *Brucella* suspension and 3 to 5 parallel streaks were made a cross of the dye plate, then incubated at 37 C° for 3 days. Bacterial growth indicated the ability of organism to resist bacteriostatic dye ^[8].

Agglutination with Monospecific Antisera:

Serological agglutination test with both monospecific antisera for *Brucella melitensis* (M) and *Brucella abortus* (A) were done by slide agglutination test. A drop of each monospecific serum (anti–M or anti–A) was added to one drop of saline suspension culture on a clean slide, mixed well with a sterile loop and

examined for agglutination which is developed within 1 min and it was compared with negative-control (one drop of saline suspension culture with one drop of distilled water). Thus, the three types of biotyping for *Brucella melitensis* were demonstrated ^[6].

Determination of *Brucella* Colonies Phase Test:

Staining of Colonies with Crystal Violet

It was carried out according to the method recommended by White and Wilson (1951) that bacterial growth was cultured on Brucella agar. The plates were incubated at 37 C° for 4 days. The growing colonies dipped in crystal violet solution for 15–20 seconds. The extra solution was drawn into sterilized solution (dettol) and neglected then the plates were examined by a hand magnifying lens near to light source. The appearance of greenish – blue color colonies indicated that the colonies are smooth. While the appearance with reddish – violet color considered evidence that they are in the rough or mucoid phase and the latter would be darker than the rough colonies.

Virulence Factors:

1: Haemolysin Production:

All bacterial isolates were streaked on blood agar and incubated at 37 C° for 24 hrs. Presence of hemolytic zone around the colonies considered as a positive result ^[9].

2: Gelatin Hydrolysis (Analysis):

Suspected colonies of *Brucella* were grown on surface of gelatin agar for 48 hrs a drop of Freezer reagent was placed on agar surface. Presences of translucent area about the colonies were considered as a positive reaction ^[10].

3: Lecithinase Production Test:

Lecithinase or egg – yolk agar was inoculated with purified bacterial isolate by streaking method on agar plate and incubated at 37 C° for 24–48 hrs. The appearance zone of thickness about of the colonies considered a positive result ^[11].

4: DNAase Production Test:

Bacterial isolate was streaked on DNAase agar and incubated at 37 C° for 24–48 hrs. The bacterial growth was covered by hydrochloric acid solution (1N). The appearance of yellow-zone around of the (colonies) was considered positive result^[12].

β-Lactamase Production Test:

Rapid iodometric method was used for the detection of β-Lactamase production as it was described by WHO (1987). The solutions were used preparation above.

Procedure:

A - Bacterial isolates were freshly prepared with a pure culture on Brucella agar.

B - A loopful of bacterial suspension was transferred to eppendorf tubes, each tube contains 100 µl of penicillin G solution and incubation at 37 C° for 30 min.

- C** - After 1 hr of incubation at room temperature, 50 µl of starch solution was added and well mix.
- D** - Added 20 µl of iodide solution and well mix. An initial blue color will rapid develop due to reaction of the iodine with starch.
- E** - Blue color was changed to white within lease than 1 min that indicated the production of β–Lactamase as positive result.
- F** - When the results (changing the color) were lettered more than 5 min the test should be returned.

Antibiotics Sensitivity Test:

Method of WHO (1984) was used in this test by using antibiotic discs above:

- 1** - Purified bacterial colonies isolated on Brucella agar were transported to test tube contains 20 ml of Brucella broth and incubated at 37 C° for 24 hrs.
- 2** - Bacterial suspension was streaked with sterile swabs on the Mueller – Hinton agar and left for 5 min, with sterile forceps the antibiotic discs were placed on the inoculated plate and incubation at 37 C° for 24 hrs.
- 3** - After incubation the diameter of inhibition zone was measured by caliper and scored as sensitive, intermediated and resistance according to criteria laid down by National Committee for Clinical Laboratory Standards (NCCLS, 1999).

Results:

Blood Samples:

Samples source	NO. of samples	No. (%) Positive Rose Bengal test.	No. (%) Positive 2 – Mercaptoethaol test
Humans	100	58 (58%)	12 (20.68%)

Table-1: Blood samples carrying as indicated by Rose Bengal test and 2–mercaptoethanol test.

Bacterial Isolation and Identification:

Brucella melitensis was isolated 8 isolates were isolated from humans blood samples, there was no significant difference in culture for isolation of *Brucella*.

After 3-5 days of incubation, the *Brucella* colonies appeared on selective medium for *Brucella* agar as pinpoint, smooth, glistening, bluish and translucent. As the age proceded the colonies became opaque and about 2–3 mm in diameter (table-2).

Species	NO. of samples Positive results of RBT and 2 - ME	Culture of cases positive
Humans	58	8 (13.79%)

Table-2: Percentage of provelance of *Brucella* recovered from blood samples by culture isolation.

Staining:

By Gram's stain and Modified Ziehl – Neelsen stain, suspected colonies showed as Gram negative rod or cocobacilli in short chain or small clusters. In modified Ziehl – Neelsen stain appear in small, red – staining cocobacilli

Biochemical Tests

Blood samples	Humans
No. Isolate by culture	8
Oxidase test	+
Catalase test	+
Urease test	V*
Indole test	-
Methyl red test	-
Voges – Proskauer test	-
Simmon citrate utilized test	-
Nitrate reduction test	+
Growth on MacConkey agar	-
Hydrogen sulfide production test	-

Table-3: Showing the result of biochemical tests of *Brucella melitensis*.

V* = variability means the result revealed positive between 4 – 24 hrs.

(-)** = Two of *Brucella* isolates of forty one gave fine growth colony on MacConkey agar.

Bacterial Growth on Bacteriostatic Dye:

All *Brucella* isolates were culture in presence of basic fuchsin dye by concentrations 1:50000 and 1:100000

Bacterial Biotyping:

Bacterial agglutination with monospecific antisera for *Brucella melitensis* (M) and for *Brucella abortus* (A) had been performed. For the eight isolates from human samples two isolates had shown agglutination positively with monospecific antisera for *Brucella abortus* (A) (25%) while 6 isolates had shown agglutination with both types of monospecific antisera for *Brucella melitensis* (M) and for *Brucella abortus* (A) (75%) (Table-4).

Species	No. of isolate	Biotype 2	Biotype 3
Humans samples	8	2 (25%)	6 (75%)

Table-4: Biotyping of *Brucella melitensis* isolates isolated from human's, goat's and sheep's blood samples.

Brucella Colonies Phase Determination Test:

All *Brucella melitensis* isolates appeared in smooth phase by greenish–blue colonies color.

Virulence Factors:

All *Brucella melitensis* isolates included this study showed no haemolysis on blood agar (Figure 6), no gelatin analysis on gelatin agar (Figure 7), no production of DNAase on DNAase agar and the isolates also showed no production of lecithinase on egg–yolk agar.

β–Lactamase Production Test:

Rapid iodometric method was used for the detection of presence of found β-lactamase production. All *Brucella melitensis* isolates exhibited negative result.

Antibiotic Sensitivity Test:

The sensitivity result of forty one isolates which isolated from blood samples of humans, goats and sheep to twenty two discs of antibiotic showed that all isolates were sensitive to Streptomycin, Pipracillin, Norfloxacin, Ciprofloxacin, Rifampicin, Doxycyclin and Cephoxitin a rate 100 %.

Also the result showed that the isolates were less sensitive to Chloramphenicol, Tetracycline and Gentamycin a rate 92%, 80% and 80% respectively. As well as resistance to Ceftriaxone, Augmentin, Amoxicillin, Nalidixic acid, Lincomycin, Trimethoprin, Cloxacillin, Cefotaxime and Clindamycin a rate 100%. (Table-5).

Antibiotic	No.*	Sensitive isolate		Resistance isolate	
		Number	%	Number	%
Cephoxitin	12	8	100	0	0
Ciprofloxacin	15	8	100	0	0
Doxycyclin	10	8	100	0	0
Norfloxacin	5	8	100	0	0
Pipracillin	6	8	100	0	0
Rifampicin	14	8	100	0	0
Streptomycin	2	8	100	0	0
Chloramphenicol	11	7	87.5	1	8
Gentamycin	1	6	75	2	25
Tetracycline	16	6	75	2	25
Amoxicillin	4	0	0	8	100
Augmentin	8	0	0	8	100
Cefotaxime	17	0	0	8	100
Ceftriaxone	20	0	0	8	100
Clindamycin	13	0	0	8	100
Cloxacillin	21	0	0	8	100
Lincomycin	18	0	0	8	100
Nalidixic acid	9	0	0	8	100
Trimethoprin	19	0	0	8	100

Table 5: Antibiotic susceptibility of *Brucella melitensis*, to different antibiotic discs.

No.* = Numbers sequence is corresponded to the number of each antibiotic disc as shown in figure 4 – 10.

Discussion:

Blood Samples:

1: Rose Bengal Test:

The whole Rose Bengal test positive samples have showed a clear agglutination. This agglutination intensity might be attributed to capability of *Brucella* to stimulate polyclonal activation of B-lymphocyte which leads to increase of antibodies titer in patient's sera ^[13]. The Rose Bengal test is considered as a survey test to investigate the infection occurrence for its high sensitivity in that it can detect the infection even with the lowest antibodies titers and detect the infection in its early stage, this is due to that the IgM antibodies are the only predominant antibodies during the acute phase of the disease and more active than IgG ^[35,7]. The test is used widely in the clinical pathology laboratory for its quick result which can be read within minutes of adding test solution on the suspected infected serum ^[14]. The test was used by many researchers who got a variable percentage as was showed by the results of Al-Mosilh et al (1989)^[15] who found on one occasion 70 positive samples out of 1019 sera (6.9%) and Mohamed and ^[16] have mentioned that the number of positive samples was 255 samples out of 986 samples (25.5%) while there was a high percentage being reported by [17] in which the number of positive samples was 100 out of 100 sera (100%) and in another study made by ^[18] it was found that the infection rate was 74.4% reflecting 146 positive samples out of 196 samples taken from people tested by Rose Bengal test.

2: Two-Mercaptoethanol Test:

This test is considered one of the important agglutination tests. Its importance come from its ability to detect the chronic phase and detecting IgG ^[19] who reported the occurrence of agglutination in each of reagent 1 and reagent 2 which indicates the presence of immunoglobulin type M or type G and this a clear indication that the infective in its chronic phase. It can be said that diagnosis the *Brucella* infection cases by serological examinations would be different and variable according to the infection stages ^[20] and this can be attributed to the difference in antibodies levels in that the level of immunoglobulin type M is increasing during the first week of acute infection and reaches its high levels during three months and it's might be detected even at low levels during the chronic infection within the treatment period, while antibodies type G increase in levels during three weeks after onset of acute infection reaching higher titer within 6–7 weeks and this titer remains at its high level even during the chronic infection ^[19].

The results of the test revealed low percentage of chronic cases because the samples was taken from newly infected animals due to the history from owners and in humans also the samples were taken from newly diagnosed patients.

Bacterial Isolation and Identification:

Samples of blood were taken from infected people and infected for isolation of bacteria because samples from lymph nodes, bone marrow, chronic suppurative

infections, synovial fluid and cerebrospinal fluid which represent the colonization location of the bacteria were not easy to be taken. In this study 8 isolates of total 58 infection cases were isolated by on Castaneda biphasic medium. This method was characterized by short time needed for isolation this could be due to liberation of bacteria from polymorphonuclear (PMN) and removal of inhibitory antibodies and even the antibiotics which might be present in the blood sample in addition bacterial concentration [21]. [22] in their study that carried in Mexico have recorded a positive result for bacterial isolation from blood reached 25.6% mean while the serological tests have shown positive result 87.9% in Rose Bengal test and 63.2% in 2–mercaptoethanol test while [21] have reported in their study in Iran that bacterial isolation rate from blood reached 16 % compared with serological tests in which a rate of 100 % has been reached for each of the following serological tests (Coomb's, 2–mercaptoethanol and standard agglutination test). Some researchers consider bacterial isolation from cases suspected of having been infected with *Brucella* is the gold standard in ascertaining the infection [9,22] while serological tests are considered as the best from the scientific point of view according to what FAO/WHO (1986)[19] had mentioned.

Microscopic and Culturing Properties:

The morphological characteristics of *Brucella* isolates were studied on selective medium for Brucella agar and after incubation periods of 2–3 days the *Brucella* colonies were characterized as pinpoint, smooth, glistening, bluish, honey colored and translucent. This was in agreement with [23]. Microscopically and after being stained with gram stain the bacteria appeared as short bacilli somewhat coccoid and gram negative, lacking the capsule, non sporforming and stained with red color when stained with modified Ziehl–Neelsen stain so with these characteristics the bacteria is typical to microscopical characteristics of *Brucella* described by [24,25].

Biochemical Tests:

All isolates were positive to Catalase, Oxidase and Urease as well as negative to Indole production, Methyl Red, Voges–Proskauer and Simmon Citrate utilization and these characteristics were agreed with characteristics reported by [24,26] regarding to *Brucella*. The *Brucella* isolates also had the ability to reduce the Nitrate to Nitrite and this agrees with [25]. In addition, among 41 isolates two only showed little growth on MacCokey agar and this result agrees with that mentioned by [8,25] that most *Brucella* isolates growth is inhibited in media containing bile salts especially MacConkey agar and all isolates have shown their inability to produce H₂S and these properties are similar to that of *Brucella melitensis*.

Bacterial Growth on Bacteriostatic Dye:

The results showed the ability of all forty one isolates to grow on media containing basic fuchsin dye in the concentrations 1:50000 and 1:100000 which

indicated the ability to resist this dye and these results agree with that mentioned by^[27,28].

Bacterial Serotyping:

Brucella melitensis isolates were determined to be biotyped by agglutination with monospecific antisera for *Brucella melitensis* (M) and with monospecific antisera for *Brucella abortus* (A).

The results of this study were in agreement with that achieved by^[29] as he obtained 15 isolates of *Brucella melitensis*, 5 of these isolates were biotype 2 and 10 isolates were biotype 3. In addition in a study of^[27] there were 15 isolates from *Brucella melitensis* of biotyping 2, 3 isolated from 100 samples taken from patients suffering for brucellosis in certain areas in Baghdad. When looking at studies made in the Arab region it appears that the present study was in accordance with^[17] who reported that *Brucella melitensis* biotype 2, 3 are the main cause of brucellosis in Saudi Arabia. While^[30] assured the increase of *Brucella abortus* infection in Yemen especially in the last years and globally it is clear that the results of this study were similar to that of^[31] who noted the increase of infection with *Brucella melitensis* biotype 3 (54.5%) in Southeast Turkey.

Brucella Colonies Phase Determination Test:

The results of this study regarding smooth phase agreed with characteristics in a study of White and Wilson, (1951)^[5].

Virulence Factors:

The results of the virulence factors were compatible with^[24,25] who have mentioned. The virulence of *Brucella* is represented by its ability to live and multiply inside macrophages selectively by inhibiting the mechanism of attachment between lysosome and phagosome^[32], while^[33,34] have mentioned that *Brucella* possesses lipopolysaccharide which contains O-chain and type IV secretory system and coproteins facilitate its growth and multiplication intracellularly and producing siderophore and the latter represents mechanisms of drawing iron from out of the cells.

β-Lactamase Production Test:

Rapid iodometric method was used to detect the β-lactamase production which depends on detecting penicillic acid or cephalosporin formed by breaking down the amide bond from β-lactamase ring in each of penicillins and cephalosporins. The iodine reacts with starch to form a dark purple (blue) complex which remains unchanged in the absence of β-lactamase secretion while when release of this bacteria enzyme the penicillic or cephalosporins reduce iodine to iodide and the latter lacks the activity of reaction with starch to form the purple complex to be converted directly to white^[35].

Antibiotic Sensitivity Test:

This test used to know the more current treatment to infections by different microorganism as well as the infection by *Brucella* for the reduced the infection and

lesser used the antibiotic and this lead to less the economic losses. Antibiotic discs diffusion method on Mueller – Hinton agar was used to check sensitivity of *Brucella* to 22 different antibiotics. The test was made to determine the most effective antibiotic to be used in treatment of the clinical cases and to limit the disease complications as possible as can and to avoid the development of bacterial resistance. Braibant, M. (2002)^[36] have recovered a protein Nor MI in *Brucella melitensis* which acts as a pump to expel drugs and toxic materials out of the bacterial cells which in turn increases their resistance to antibiotics. Our results were in agreement with ^[37] who that use repeated of Streptomycin, Tetracyclin, Gentamicin, Doxycyclin and Tobramycin were sensitive in percentage 100% as well as less sensitivity to Chloramphenicol, Neomycin, Cephalexin and Cephoxitin; and also observed that all isolates were resistant to Lincomycin and Polymexin B at a rate 100% The results were similar to ^[1] who stated that all isolates were sensitive to Streptomycin, Tetracycline and Gentamycine at a rate of 100% and Chloramphenicol (82.3%) but they were resistant to Nalidixic acid and Penicillin G at a rate of 100% The results of the current study were in contrast with ^[38] who observed that all bacterial isolates were resistant to Streptomycin, Trimethoprin at rate 100%. Depending on that it can be said that the antibiotics of choice were Streptomycine, Rifampicin, Doxycyclin, Pipracillin and Ciprofloxacin. Regarding this fact ^[39,40,4] have showed the importance of using two antibiotics such as using Doxycycline with Streptomycin or Rifampicin for a period not less than six weeks to control the disease in its acute phase and to avoid the complications and/or the relapse as well. There are many important factors affecting the results of antibiotic sensitivity test such as virulence of the isolate, volume of the inoculate, growth intensity on surface of culture plate, type of medium and PH of medium ^[41].

Conclusions:

According to our study and results achieved we can conclude the following:

- 1 - Infection with *Brucella melitensis* (biotype 3) was the most common followed by *Brucella melitensis* (biotype 2).
- 2 - The study results have showed that all local isolates have given a negative result for β -lactamase by using rapid iodometric method.
- 3 - The study indicated that the local isolates showed sensitivity 100% to Streptomycin, Pipracillin, Norfloxacin, Ciprofloxacin, Rifampicin, Doxycyclin and Cephoxitin. Thus they can be use in treatment of the disease.
- 4 - The study results have showed that the infected rate in human cases the female is higher than in the male.
- 5 - The serological tests especially 2 – mercaptoethanol gives a clear idea of the disease phase compared with Rose Bengal test which gives an idea about the acute and chronic phase.
- 6 - The study showed efficiency of using isolation on Castaneda biphasic medium

Recommendations:

According to results of our study we recommend the following:

- 1 - Conducting an awareness programs for peoples refer to disease importance its transmission ways and its control measures.
- 2 - Doing more studies to detect type of antigens isolated from local *Brucella* to be used in ELISA test to diagnose Brucellosis.
- 3 - The identification of bacteria by using Polymerase Chain Reaction (PCR).

References:

- 1 - Abed–Mohammed, K. I. (1998). Immunological, Biochemical and Bacteriological study of *Brucella* disease in human. PH. D. thesis., Collage of Science, University of Al –Mustansiriya.
- 2 - Akova, M; Gur, D.; Livermore, D. W.; Kocagoz, T. and Akalin, H. E. (1999). In vitro activities of antibiotics alone and in combination against *B. melitensis* at neutral and acidic pH. Antimicrobial Agent and Chemotherapy., 43 (5) :1298 1300.
- 3 - Christie, A. B. (1980). Infectious Disease, Epidemiology and Clinical practice. 3rd Ed. Churchill Livingstone.
- 4 - Jones, L. M. (1967). Report to the international committee on no men culture of bacteria by the sub – committee on taxonomy of *Brucella*. Inter. J. Systemic Bact., 17: 271 – 75.
- 5 - Alton, G. G.; Jones, L. M. and Pietz, D. E. (1975). Laboratory techniques for the Brucellosis 2nd ed. Monogr. Ser. No. 55. World Health Organization. Geneva. Switzerland.
- 6 - Alton, G. G.; Jones, L. N.; Angus, R. D. and Verger, J. M. (1988). Techniques for the Brucellosis Laboratory. Institute national De La recherché agronomique 147, rue de l'university. 75007. Paris.
- 7 - Coghlan, J. D. (1997). Medical Microbiology. A guide to microbial infection: Pathogenesis, immunity laboratory diagnosis and control, 15th Ed. Churchill Livingstone.
- 8 - Corbel, M. J.; Jill, K. P. and Thomas, E. L. (1978). Method for the identification of *Brucella*. Ministry of Agriculture, Fisheries and Food Werbridge, U.K.
- 9 - Macfaddin, J. F. (2000). Biochemical Tests for Identification of Medical Bacteria. 3rd Ed. Lippincott Williams and Wilkins USA.
- 10 - Merchant and Packer. (1976). Veterinary Bacteriology and Virology – th Ed. The Iowa state University press, Ames, Iowa, USA.
- 11 - Lanyi, B. (1987). Classical and Rapid Identification Method for Medically Important Bacteria. V19 Academic Press Limited.

- 12 - Finegold, S. M. and Baron, E. J. (1986). Methods for testing antimicrobial effectiveness in Baily and Scotts diagnostic microbiology. 7th Ed. The C. V. Mos. By Co. West line. Industrial Drive, St., Louis, Missouri, USA.
- 13 - Goodwin, J. S. (1985). Prostaglandins and Immunity 1st Ed. Martinus, Nijhoff Publishing USA., P.:69 – 90.
- 14 - Daddod, W. A. and Abdulia, Z. A. (2000). A panel of eight tests in the serodiagnosis and immunological evaluation of acute brucellosis. East. Med. Health. J., 6: 304 – 12.
- 15 - Al – Mosilh, M. I.; Al – Mulle, Z. M. and Al – Samarae. S. S. (1989). Seroepidemiological studies of brucellosis in cases of fever of unknown origin in Baghdad. Iraq. Med. J., 38 : 13 – 20.
- 16 - Mohamad, N. R. S. and Al – Nasiry, S. A. (1996). Brucellosis in Iraq, Seroepidemiologic study. J. Comm. Med. Iraq. 9 (2): 202 – 6.
- 17 - Shaalan, A. A.; Memish, Z. A.; Mohamoud, S. A.; Alomori, A.; Khan, M. Y.; Almuneef, M. and Alalola, S. (2002). Brucellosis in children: Clinical observation in 115 cases. Int. J.Infect. Dis., 6 (3): 182 – 6.
- 18 - Salih, A. M. (2000). Evaluation of Brucellosis in Baghdad city. M. Sc. Thesis, College of Education. University of Al – Kadisia.
- 19 - Brooks, G. F.; Butel, J. S. and Morse, S. A. (2004). Medical Microbiology. 23rd Ed. Jawetz, Melnick, Adelberg's. New York. Mc Graw Hill : 284 – 286.
- 20 - Araj, G. F. (1986). Evolution of ELISA in the diagnosis of acute and chronic brucellosis in human beings. J. Hyg. Camb., 97 :45 – 69.
- 21 - Hajia, M. and Rahbar, M. (2006). Isolation of *Brucella* from blood culture of hospitalized brucellosis patients. Iran. J. Clin. Infect. Dis., 1 (2): 5 – 10.
- 22 - Godfroid, J. and Kasbohrer, A. (2002). Brucellosis in European union and Norway at the turn of the twenty – first century. Vet. Microbiol., 90: 135 – 45.
- 23 - Greenwood, D.; Slackand, R. and Peutherer, J. (1997). *Brucella, Bartonella* and *Strepto bacillus*. Medical Microbiology 5th Ed. Churchill Livingstone, London. P.: 325.
- 24 - Holt, J. G.; Krieg, N. R.; Sneath, P. H.; Staley, J. T. and William, S. T. (1994). Bergey's Manual of Determinative Bacteriology 9th Ed. William and Wikins Co. Baltimore. London. P: 79.
- 25 - Gilligan and York. (2004). Sentinel Laboratory Guide lines for suspected agents of Bioterrorism *Brucella* species American Society for Microbiology.
- 26 - Noviello, S.; Gallo, R.; Kelly, M.; Limberger, R. J.; Deangelis, K.; Cain, L.; Wallace, B. and Dumas, N. (2004). Laboratory – acquired brucellosis emerging. Infect. Dis., 10 (10): 1848 – 50.
- 27 - Al – Thawani, A.; Al – Bayatti, S.; Abass, A. and Abdul – Hussin, T. (2000). A study in the Epidemiological of Brucellosis in some production animals in the Province of Baghdad. The Vet. J., 10 (1): 168 – 174.

- 28 - Al – Nuaimi, A. E. S. (2007). Study of some aspects serological and genetic from a local isolates of the *Brucella* spp., Ph. D. Thesis., College of Science, University of Al – Mustansiriya
- 29 - Al – Erqwazi, Y. B. R. (1995). Bacteriological and immunological study for Brucellosis in clinical cases to human. M. Sc. Thesis. College of veterinary medicine. University of Baghdad.
- 30 - Foster, G.; Osterman, B. S.; Godfroid, J.; Jacques, I. and Cloeckaert, A. (2007). *Brucella ceti* sp. nov. and *Brucella pinnipedialis* sp. nov. for *Brucella* strains with cetaceans and seals as their preferred hosts. Int. J. Syst. Evol. Microbiol., 57: 2688 – 93.
- 31 - Nomidurn, M.; Gungor, K.; Dikensoy, O.; Ekinici, E.; Karaoglan, I. and Bekir, N. A. (2003). Epidemiological, Clinical and Laboratory features of brucellosis a prospective evaluation of 120 adult patients. Int. J. Clin. Pract., 57 (1): 20 – 24.
- 32 - Maurin, M. and Raoult, D. (2001). Use of aminoglycosides in treatment of infections due to interacellular bacteria. Antimicrob. Agents and Chemother. 45 (11); 2977 – 86.
- 33 - Rouot, B.; Teresa – Alvarez, M.; Marius, C.; Menanteau, P. and Guilloteau, L. (2003). Production of Type IV secretion system differs among *Brucella* species as revealed with B5 – and Vir B8 – specific antisera. Infect. Immunit., 71 (3): 1075 – 82.
- 34 - Lapaque, N.; moriyon, I.; Moteno, E. and Gorvel, J. P. (2005). *Brucella* lipopolysaccharide acts as a virulence factor. Curr. Opin. Microbiol., 8 (1): 60– 66.
- 35 - Morgan, W. J. (1967). The serological diagnosis of bovine brucellosis. Vet. Rec., 80: 612 – 20.
- 36 - Braibant, M.; Guillotrau, L. and Zygmunt, M. S. (2002). Functional characterization of B. melitensis Nor MI, an efflux pump belonging to the multidrug and toxic compound extrusion family. Antimicrobial Agents and Chemotherapy., 46 (9): 3050 – 53.
- 37 - Korji, S. H. A. (1991). Distribution of *Brucella* which Isolates from milk and cheese in Baghdad region. M. Sc. Thesis. College of Science. University of Al– Mustansiriya.
- 38 - Al – Abbasi, A. M.; Alwan, S. J. and Al – Jubbory, S. F. (1991). Brucellosis in Baghdad a study of 64 cases. Iraqi J. Microbiol., 3 (1): 34 – 41.
- 39 - Naroeni, A.; Jouy, N.; Ouahrani – Bettache, S.; Liautard, J. P. and Porte, F. (2001). *B. suis* impaired specific recognition of phagosomes by lysosomes due to phagosomal membrane modifications. Infect. Immun., 69 (1): 486 – 93.
- 40 - Pappas, G.; Akritidis, N.; Bosilkovaski, M. and Tsianose, E. (2005). Brucellosis. N. E. J. M., 352 (22):2325 – 36.

- 41 - Solera, J.; Rodriguez – Zapata, M.; Gijo, P.; Largo, J.; Saez, L. and Martinez, A. (1995). Doxycycline – rifampin versus doxycycline – streptomycin in treatment of human brucellosis due to *B. melitensis*. *Antimicrob. Agents Chemother.*, 39 (9): 2061 – 67.
- 42 - Freeman, B. A. (1985). *Text Book of Microbiology*. Chap. 22. W. B. Saunders company.
- 43 - Refai, M. (2002). Incidence and control of brucellosis in the near east region. *Vet. Microbiol.*, 90 (1 – 4): 81 – 110.
- 44 - Shanshal, R. Z. S. (1999). Epidemiological study of *Brucella* in Baghdad M. Sc. Thesis, College of Veterinary medicine. University of Baghdad.
- 45 - Sykes, R. B. and Mathew, M. (1976). The β – lactamase of gram – negative bacteria and their role in resistance to β – lactam antibiotics. *J. Antimicrob. Chemother.*, 2: 115 – 57.
- 46 - Gandara, B.; Zheludkov, M. M. and Chernysheva, M. I. (1994). An evaluation of the effectiveness of laboratory diagnostic methods for brucellosis. *Zh – MiKrobiol. Epidemiol. Immunobiol.*, 4: 55 – 58.
- 47 - Turkmani, K.; Ioannidis, A.; Christidou, A.; Psaroulaki, A. and Tselentis, Y. (2006). In vitro susceptibilities of *Brucella melitensis* isolates to eleven antibiotics. *Ann. Clin. Microbiol. Antimicrob.*, 5 (24): 332 – 35.
- 48 - World Health Organization (WHO) (1984). Report of WHO consultation of veterinary public health: aspect of prevention and control of *Campylobacter* infections. Moscow, 20 – 22 February.
- 49 - World Health Organization (WHO) (1987). Techniques for the detection of β – lactamase producing strains of *Neisseria gonorrhoeae*. 616: 137 – 43.