BACTERIOLOGICAL STUDY ON PREVALENCE OF *Brucella melitensis* IN SMALL RUMINANTS AT DIYALA GOVERNORATE

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ABSTRACT

This work aimed at detecting prevalence of *Brucella* infection in flocks of sheep and goats with previous and recent history of abortion and decreased fertility in certain districts of Diyala Governorate. A cross sectional bacteriological study was conducted from September/2016 to June/2017. Tissue samples as placentas, fetuses and vaginal swabs were collected from aborted does and ewes; vaginal swabs were taken from females at random and from those with previous history of abortion, still birth and infertility. Using Stamp stain; acid fast, bright red coco-bacilli were detected in primary smears prepared from 27 (39.70%) out of 68 tissue samples (placentas, fetuses and vaginal swabs from sheep and goats). Meanwhile, *Brucella* spp. was isolated in 2 (4.54%), 1 (33.3%) of the vaginal discharge, placentas, respectively and none isolation from fetal stomach contents. Growth characteristics stain reaction and bacterial morphology indicated that the isolation was *Brucella* species; while results of biochemical activities and biotyping assays showed that the three isolated strains of *Brucella* in this study were *B. melitensis* biotype 1. Result obtained in this study indicated that *B. melitensis* biovar. 1 is prevalent in this Governorate and it is a main cause of abortion and infertility in small ruminants; as no other known cause of abortion was detected during the bacteriological study.

Key words: Isolation, Botyping, *Brucella melitensis*, abortion, sheep, goats, Diyala.

INTRODUCTION

Brucellosis is a general term used for animal and human infections that is caused by several species of the genus *Brucella*, mainly *Brucella abortus*, *Brucella melitensis* and *Brucella suis* (OIE, 2016). *Brucellae* are Gram-negative, weakly acid-fast, facultative intracellular coccobacilli (Gwida *et al.*, 2010). *Brucella melitensis* is the most virulent and most common cause of human brucellosis worldwide (Blasco and Molina-Flores, 2011). Infection with *Brucellae* is still one of the most important and widespread zoonosis in the globe

according to reports of FAO, WHO and the OIE organizations (Lopez *et al.*, 2010). Brucellosis is a highly infectious, re-emerging bacterial disease of man and animals (Hadush and Pal, 2013). *Brucella* infection in animals is readily transmissible to humans by consuming undercooked meat or unpasteurized/raw dairy products. Inhalation of aerosols harboring the bacteria and through skin wounds or mucous membranes is also considered. Laboratory acquired infection with *Brucellae* is so common and it has been reported worldwide (OIE, 2016); cases of laboratory acquired brucellosis represents about 20% of all human infections. Brucellosis is characterized by acute febrile illness which may progress to a more chronic form which is severely debilitating and disabling illness, (OIE, 2016), it is still endemic in the Mediterranean basin, Middle East, Western Asia, Africa, and South America (Maurine, 2005). Small ruminant's populations in these regions showed seroprevalence values that are among the highest worldwide (Musallam *et al.*, 2016). There are about half million new human cases of brucellosis reported annually worldwide, making it the most common zoonosis (Saleem *et al.*, 2010). Brucellosis is enzootic and endemic in Iraq since 1937, and it was first isolated by an Iraqi physician (Al-Zahawi, 1938; Saleem et al., 2010). An accurate diagnosis of brucellosis is important for the control of the disease in animals and consequently in man. As members of the genus *Brucella* are slow growing and highly fastidious (Al-Dahuok *et al.*, 2003); serological tests are considered the most useful and commonly used tool for the diagnosis of Brucellosis in man and animals; as they are cheap, fast and safe. Nevertheless; isolation and identification of *Brucella* spp. is still considered the 'gold standard' for the diagnosis of brucellosis in animals although it requires high security laboratory facilities and it takes at least a week to be completed (Nielsen and Yu, 2010). In Diyala province, there are few studies concerning human and animal brucellosis. (Qasim *et al.*, 1995 ; Al-Dileamy, 2010 ; Fadihl and Khalil, 2016).

This study aimed to diagnose bacterial causes of abortion with special reference to *Brucellae* in small ruminants; through bacteriological examination of aborted fetuses, placentas and vaginal discharge of recently aborted ewes and does in some areas of Diyala province. This is mostly carried to evaluate the importance of this infectious zoonotic disease in this Governorate.

MATERIALS AND METHODS

Area of the study

This cross-sectional study was carried in the period extending from September 2016 to June 2017. It was conducted on ewes and does present in

some districts of Diyala Governorate; including: Qara- Tabbah, Bardiya, Al-Anbakya, Door Mandali and Kan Bani Saad. These geographical areas were chosen according to previous and recent information of reproductive problems as late abortion, stillbirth, and infertility in sheep and goats.

Collection of samples

Vaginal swabs: Sixty three vaginal swabs were collected from aborted females and other females at random during period of the study using sterile cotton swab (Afco ®) with 2 ml of TSB (Trypticase soy broth) as transport medium. Swab was aseptically removed from its tube and introduced into the animal vagina.

Aborted fetuses: Two ovine aborted fetuses.

Placentas: Three retained placentas with cotyledon and fetal sac were collected from different flocks.

Milk samples: Ten milk samples were aseptically collected from both quarters of each animal.

All collected samples were kept coole until delivered to the laboratory or kept frozen till processed.

Bacteriological examination

Bacteriological processing of tissue samples and biochemical identification of isolates were performed at Clinical Pathology Lab./ Dep. Internal and Preventive Medicine/ College of Veterinary Medicine/ University of Diyala. Complete identification and biotyping were completed at Central Veterinary laboratories / Ministry of Agriculture/ Baghdad.

Culture of specimens

Processing of tissue samples was carried according to Alton and colleagues (Alton *et al.*, 1986). All samples were inoculated on duplicate of blood agar (BA) and Trypticase soy agar (TSA) plates using dilution method to obtain separated colonies for purification. Inoculated plates were incubated at 37 ˚C both in aerobic and microaerophilic conditions using a candle jar system (OIE, 2016). Plates were inspected for appearance of growth after 2-7 days of incubation.

Direct tissue smears examination

Impression smears were prepared from all vaginal swabs, cotyledons and fetal stomach contents (68 samples); they were dried in air, fixed on flame, stained by Gram, s and Modified acid fast stain (Stamp stain) applied as suggested by (Alton et al., 1988).

Macroscopical and Microscopical examination of bacterial colonies

Bacterial growth on solid media was examined when appeared, checked for purity and for appearance on media with or without CO_2. Colony morphology regarding size, color, translucency, smoothness, presence of hemolysis, edge appearance and colony elevation were recorded. Gram and Stamp stains were used for microscopical examination of colonies suggestive to be for *Brucella* or other causes of abortion. Interesting colonies and those yielding Gram negative, acid fast coccobacilli were sub cultured on Blood agar (BA), TSA and incubated as previously described to obtain pure isolates.

Identification of *Brucella* species

Biochemical test

Bacterial isolates were subjected to classical biochemical tests characteristic for the genus; as production of catalase, oxidase, urease and indol, in addition to ability to grow on Simmons citrate agar, MacConkey agar and nitrate reduction (Alton *et al.*, 1988 ; OIE, 2016).

Differentiation of vaccine and field strains

Vaccine and field strains were differentiated by growing bacterial isolates on TSA medium; part with penicillin in a concentration of 5 IU ml^{-1} of media and another part supplemented with streptomycin in a concentration of 2.5 µg ml^{-1} of media (Alton *et al.*, 1988).

Bio- typing of Brucella isolates

Species and biotyping of isolated Brucella strains were carried according to the following: CO_2 requirement for growth, production of H_2S, urease, agglutination with mono specific anti Brucella sera (Anti-A and Anti-M), and growth in the presence of dyes (basic fuchsine 1:100.000, thionin 1:100.000) (Al-Dahouk *et al.*, 2003).

RESULTS

Direct smears using Stamp stain

Using Stamp stain, acid fast, bright red coco-bacilli were detected in primary smears prepared from 27 (39.7%) out of 68 placenta and vaginal swab from sheep and goats, other bacteria appeared blue in color. Most red bacteria suggestive to be a *Brucella* spp. were extra-cellularly located; only few were intracellular (Fig. 1). Smears from stomach contents of aborted fetuses were negative for all expected causes of abortion (Table 1). Characteristic morphology for other bacterial causes of abortion as *Listeria monocytogenes*,

Campylobacter spp., *Coxiella burnetii* or *Chlamydia abortus* were not detected in Gram or Stamp stained direct smears.

Bacterial isolation

Three *Brucella* spp. were isolated from a total of 68 (4.41%) tissue samples (VS, fetuses and placentas) collected from ewes and does (Table 1), milk samples yielded no bacterial growth. Bacterial colonies typical for *Brucella* species appeared after 3 days of incubation; both at aerobic and microaerophilic conditions (most cultures were not pure and have contaminants). On blood agar, colonies suspected to be for *Brucella* were small (about 0.5-1mm in diameter) (Fig. 1); then they increase in size by further incubation, they were non hemolytic, moderately convex, smooth, glistening with evenly rounded margins; when viewed from above, colonies appeared smooth and pearly grayish- blue-white in color. On TSA medium, colonies were off white in color, small, smooth, glistening and translucent. Gram and Stamp stained smears prepared from suspected colonies yielded Gram negative, extremely short rods; while Stamp stained smears revealed bright red cocoid rods appearing bright against a blue back ground (Fig. 2), bacteria other than *Brucella* appeared blue in color. Identified colonies were sub cultured on BA & TSA and were aerobically incubated for further identification. None of the tissue samples cultured from does yielded bacterial growth suggestive for *Brucella*.

Table 1. Positive cases of *Brucella* using direct smears and culture from ewes and does

Type of sample	No of samples	Positive direct smear	Isolated *Brucella*
Vaginal swab (ewes)	44	21 (47.72 %)	2 (4.54 %)
Placenta (ewe)	3	3 (100%)	1(33.3%)
Aborted fetus (ewe)	2	Negative	Negative
Vaginal swab (does)	19	3 (15.87%)	Negative
Total number	68	27 (39.70%)	3 (4.41%)

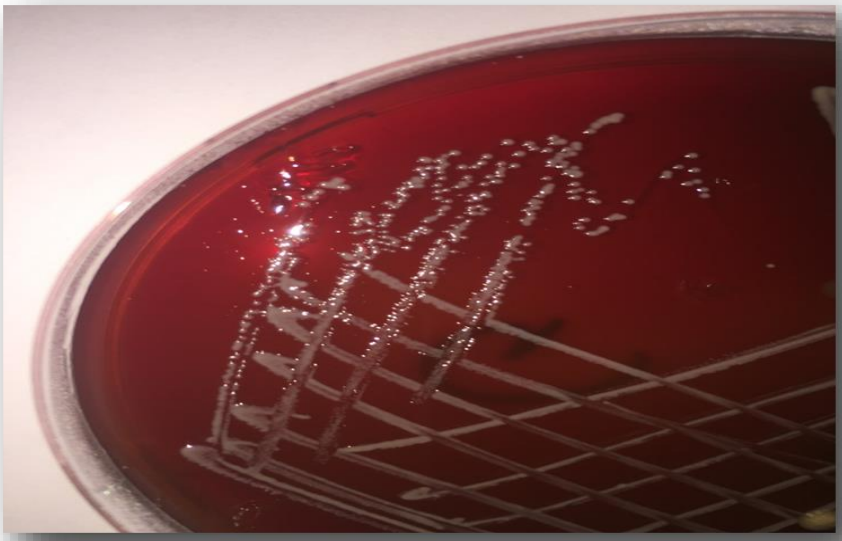

Fig. 1. Small, smooth, glistening colonies of isolated *Brucella* Spp. on Blood agar

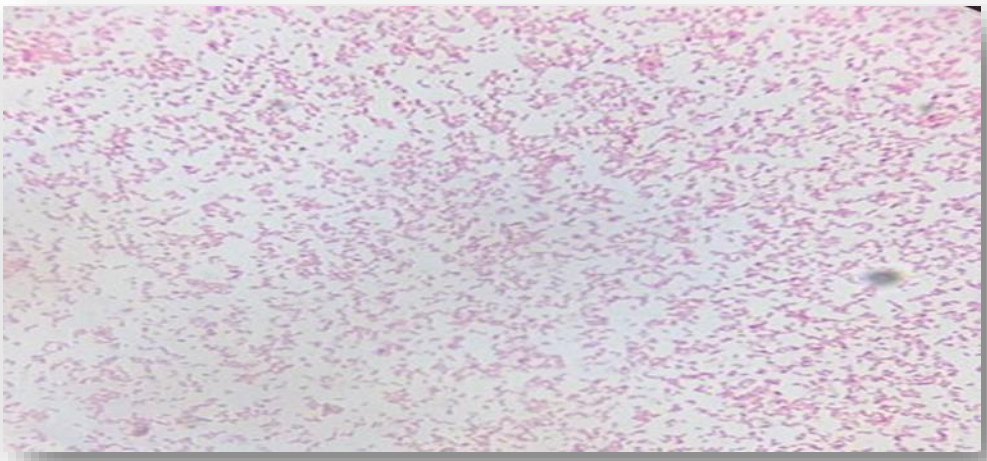

Fig. 2. Acid resistant red short rods using modified acid fast stain (Stamp stain) from colonies on Blood agar (100X)

Species identification and biotyping of *Brucella* isolates

Brucella isolates were primarily identified on genus level depending on slide morphology, staining reactions and biochemical activities as production of catalase, oxidase and urease enzymes, nitrate reduction, inability to utilize citrate to grow on Simmons citrate agar in addition to failure to grow on MacConkey agar (Table 2). In addition, the three isolated strains grew without the need for CO_2 in primary isolation, they did not produce H_2S, grew well in media with thionin and basic fuchsine and all showed agglutination with monoclonal anti *B. melitensis* antiserum (M). The three isolated strains grew on media containing penicillin and not in that containing streptomycin which differentiated the

isolated species from Rev-1 living vaccine strain (Table 3). All previously obtained data indicated that the two isolates were *B. melitensis* bio var.1.

Table 2. Results of biochemical reactions of isolated *Brucella* strains

Isolate No	CO_2 requirement	Oxidase	Urease	H_2S	Growth on MacConkey	Nitrate reduction	Citrate Utilization	Indol	Catalase
1*	-	+	+	-	-	+	-	-	+
2*	-	+	+	-	-	+	-	-	+

*1 means one isolate of *Brucella* melitensis /2* means two isolates of *Brucella melitensis* from VS and cotyledons of the same animal.

Table 3. Result of Bio-typing tests for *Brucella* isolates

Isolate No	Thionin (40 µg ml^{-1}) 1:100,000	Basic fuchsin (40 µg ml^{-1}) 1:100,000	Streptomycin (2.5 µg ml^{-1})	Penicillin (5 IU ml^{-1})	Agglutination mono specific antisera A	M
1*	+	+	-	+	-	+
2*	+	+	-	+	-	+

A: anti-*Brucella abortus*/ M: anti-*Brucella melitensis.**1 means one isolate of *Brucella* melitensis. 2* means two isolates of *Brucella melitensis* from VS and cotyledons of the same animal.

DISCUSSION

To conduct bacteriological examination on aborting materials; only small numbers of samples could be obtained during this study; and this may partly explain few *Brucella* strains isolated (3%). It was not easy to collect Placentas and aborted fetuses because farmers are used to make them a delicious meal for their dogs. Most of the cotyledons obtained from aborted animals were autolyzed as they were removed from animals by veterinarians to treat cases of retained placentas following abortion or normal parturition, which is commonly considered as an indication for animal brucellosis.

The first observation suggested the presence of *Brucella* was provided by detecting acid fast, *Brucella*-like organisms in smears prepared from VS and placentas of affected animals using modified acid-fast stain "Stamp's stain" (Alton *et al.*, 1988). This finding has long been considered sufficient to give a primary presumptive diagnosis for this disease in animals (OIE, 2016). The presence of *Coxiella burnetii* and *Chlamydia abortus* that may apparently resemble *Brucella* has been excluded; since most of the red cocobacilli detected in each positive smear were extracellularly located which is not the picture with the two previously mentioned obligatory intracellular bacteria (Alton *et al.*, 1988).

Failure of isolating *Brucella* from the few milk samples included in this study may be explained by limited number of samples collected, as most of the examined ewes and does were dry. Anyhow, *Brucellae* are known to be only intermittently secreted in milk of infected animals and successive milk samples from the same animal are needed to achieve a successful isolation for *Brucella* species. In addition to that colonization of the udder with *Brucella* spp. is associated with subclinical mastitis and great reduction in milk yield due to chronic inflammatory changes in the infected mammary gland and that explains the scarcity in milk of studied flocks. It was also reported that some *Brucella* seronegative females were found to shed *B. melitensis* in milk postpartum, whereas others do not shed *Brucellae* despite being infected. It is worth noting that in sheep, chronic brucellosis is seldom accompanied by prolonged excretion of the bacteria (Godfroid *et al.*, 2011).

This finding agreed with that obtained by Al-Bakri *et al.*, (2016); where *Brucella* was isolated from only 6 out of 87 milk samples (6.89%) (Al-Bakri *et al.*, 2016), in another study conducted in (2013) by Al-Abdaly *et al.*, on Brucellosis in ruminants and humans in Nineveh Province; *Brucella* sp. was isolated from 4.2% (3 out of 73) of cheese samples made from local ewe milk, while from milk samples 2.33% (1 out of 30 samples) showed bacterial growth; and this is considered a low percentage for isolation, while in the same study. *Brucella* spp. was isolated from most aborted fetuses and fetal membranes (Al-Abdaly *et al.*, 2013), the high percentage of *Brucella* isolates in their study indicated that samples used were fresh and viable bacteria are still present.

Ignoring the use of selective media in isolating *Brucella* in this study was because of its known inhibitory action for some strains of *B. abortus, B. melitensis and B. ovis* (Poester *et al.*, 2010). In addition to that, certain antibiotics included in this media were not available at the time of conducting this study.

Although about 47.7% of direct smears prepared from VS gave a positive result for presence of acid fast, *Brucella* like bacteria, bacterial isolation was successful only in 3% of total samples. This may be explained by competition of *Brucella* with fast growing contaminants and normal flora of vagina for nutrients in media. Moreover, number of *brucellae* excreted through vagina decreases gradually by passing of time after parturition or abortion, and vaginal swabs taken immediately after abortion are the ideal source of *Brucella* when the fetus and placenta are not available (Poester *et al.*, 2010). In general, the chance for *Brucella* isolation gradually became limited as most excreted bacteria

became nonviable. Anyhow, this observation was expected and bacterial isolation is not always capable of confirming a preliminary diagnosis of brucellosis (Poester *et al.*, 2010).

Depending on results of characteristic biochemical and biotyping tests (Alton, 1988 ; OIE, 2016); the three isolated strains (Two strains were isolated from VD and placenta of the same animal) were *B. melitensis* biovar. 1. Rev.1 vaccine strain (which is in current use in Iraq) is not attenuated enough and it can cause brucellosis in vaccinated animals and man (Issa, 2013). In addition to that it has similar properties to *B. melitensis* biovar 1 which has been identified in this study; so the isolated field strains were differentiated from the vaccine strain by their ability to grow on media containing thionin, basic fuchsia and penicillin but not on those with streptomycin, while Rev 1 vaccine strain can grow on media containing streptomycin but not on media with thionin, basic fuchsin or penicillin (Alton *et al.*, 1988). A study conducted in Diyala Governorate, on prevalence of *Brucella* species in local cheese prepared from unpasteurized milk, *Brucella* spp. was detected in 12% of cultured samples, 8% was *B. melitensis* the rest 4% were identified as *Brucella abo*rtus (Fadihl and Khalil, 2016), biotyping for isolated species was not carried.

Through revising literatures*; Brucella melitensis* biovar 3 seems to be the most prevalent biotype in countries of the Middle East as Egypt, Jordan, Occupied Palestine, Turkey, Maghreb region (Algeria/Morocco/Tunisia), Mediterranean region, Central Asia and parts of Latin America where brucellosis is still persistent among sheep, goats and humans. In addition to Iraq, *B. melitensis* biovar 2 has been reported in Turkey, Iran and Saudi Arabia, and *B. melitensis* biovar 1 in Libya, Oman and Occupied Palestine (Lounes et al., 2014).

Isolation of *B. melitensis* biovar1 in this study agreed with some studies conducted on brucellosis in small ruminants in other provinces of Iraq and in neighboring countries (Young and Corbel, 1989). In AL-Najaf province the 3 biovarieties of *B. meltitensis* were isolated from aborted materials of ewes but biovar 1 showed the highest number isolated (50%) while biovar 3 was the least prevalent (Al-Tememy *et al.*, 2013).

Results reported in the current study disagreed with that obtained in other provinces of Iraq, in Baghdad; it was found that *B. melitensis* biovar 3 was the only biovar isolated from aborting sheep and goats (Saleem *et al.*, 2004). While in Al-Basrah province, *B. melitensis* biotype 2 and 3 were identified in raw milk of cows in addition to 33 *Brucella abortus* (biotypes 2, 3, 4 and 6); and 4 *B. ovis*

(Abbas and Al-Deewan, 2009), in 2010, *B. melitensis* biotype 2 has been isolated from milk products and biotype 1 was not detected (Abbas and Talei, 2010). In Kut- Wasit province *B. melitensis* biovar 3 was isolated from blood of humans, goats and sheep with clinical history suggestive for Brucellosis (Tofah, 2008).

Meanwhile, *B. melitensis* biotype 1 and 2 were detected in blood of human patients with rheumatological manifestations in a hospital in Wassit province (Nidhal *et al*., 2012). Anyhow, it is well known that in developing countries where *B. melitensis*, is the most important cause of human brucellosis; biovars 1 and 3 are the most prevalent (Benkirane, 2006).

Diyala Governorate has long eastern borders with the Iranian West; and *B. melitensis* has long been known as a cause of brucellosis in small ruminants and man in Iran; although the three biovar of this species were found there but biovar 1 is the more prevalent and widely endemic according to veterinary authorities (Bahmani *et al.*, 2017). *Brucella melitensis* has been reported for causing abortion in Iranian cattle, the three biovar were detected but biovar 1 was the more prevalent in cattle and man, in a more recent bacteriological study on *Brucella* suspected human cases, *B. melitensis* biovar 1 was the only biovar detected (Erami *et al*., 2016).

Shared borders between Iraq and Iran; allows free movement of animals across the two countries and this may in part explain high prevalence of biotype 1 in animals of this province. In addition to that, Iraq imported large number of Iranian livestock including sheep and goats that are entering different provinces without being subjected to clinical or laboratory examination by Iraqi veterinary authorities for their being free from infectious disease as brucellosis.

Brucella melitensis biovar 1 is also considered the most common *Brucella* species detected in Sultanate of Oman (El Hag El Tahir, 2011), in India, *B. melitensis* biovar 1 is the most predominant (Barua *et al.*, 2016) and Spain (Colomenero *et al.*, 1996). While in Abu Dhabi- Emirates, *B. melitensis* has been isolated for the first time from milk of cattle, sheep, goats and camels raised in the region; most biotypes were biotype 3 followed by biotype 1 (Myoma *et al.*, 2014).

In Kuwait, *B. melitensis* biovar 2 was isolated from aborted fetuses of lactating cattle (El-Gohary *et al.*, 2016). This biovariety has also been reported in Al-Basrah province, which has shared borders with Kuwait (Abbas and Al-Deewan, 2009). On the other hand, in the Kingdom of Saudi Arabia (KSA) sharing long borders with the South of Iraq, Brucellosis is endemic and *B.*

melitensis biovar 1 was previously found to be the most widely isolated *Brucella* from humans in Riyadh followed by biovar 3 (Qadri *et al.*, 1989); later *B. melitensis* recorded in humans in the Asir and Medina regions were biotype 3 (Al-Sekait, 2000).

On the other hand, in Jordan, partially bordering East of Iraq and where brucellosis is an endemic disease; *B. melitensis* biovar 3 has been reported as a cause of massive abortion in sheep and goats (Al-Ani *et al.*, 2004). In a more recent study; it was found that *B. melitensis* bio 1 is most predominant among *Brucella* infected children in Jordan (Magableh and Bataineh, 2007). Iraq lying to the South of Turkey and *Brucella* has long been endemic in this country where most *Brucella* spp. isolated from small ruminants was found to be *B. melitensis* biovar 3 (Tuba *et al.*, 2012).

In other parts of the world; *B. melitensis* biovar 1 was found to be the most prevalent *Brucella* species among humans and small ruminants in Peru, the South American country where Brucellosis is still endemic (Nöckler *et al.*, 2009). The same is true for brucellosis in China where it is endemic in certain localities and it is caused by the three biovar of *B. melitensis* but since 2005, most human cases in China have been caused by *B. melitensis* biovar 3 (Xiao *et al.*, 2015).

In Egypt, *B. melitensis* biovar 3 is the most prevalent strain isolated from sheep, goats, cattle in addition to camels in different parts of this country (Mona, 2015). In 2015, *B. melitensis* biovar 1 was reported for the first time from sheep in Sudan (Ahmed and Musa, 2015).

CONCLUSION

1. Brucellosis is endemic in small ruminants in Diyala Governorate.
2. *Brucella melitensis* biovar- 1 is the only bacterial pathogen identified in aborting materials included in this study.

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دراسة بكتريولوجية لانتشار البروسيلا مليتنسز في المجترات الصغير في محافظة ديالى

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المستخلص

هدف هذا العمل لدراسة مدى انتشار الاصابة بجرثومة البروسيلا (*Brucella*) في الاغنام والماعز في بعض مناطق محافظة ديالى من خلال اجراء دراسة مقطعية جرثومية ومصلية للفترة من شهر ايلول 2016 ولغاية شهر حزيران 2017. جمعت عينات متمثلة بالاجنة والمشائم المجهضة مع مسحات مهبلية من اناث الماعز والاغنام المجهضة، كما جمعت مسحات مهبلية من الاناث التي لديها تاريخ قريب بحصول اجهاضات، ولادات ميته مع تراجع في الخصوبة، كذلك جمعت مسحات مهبلية من الاناث بشكل عشوائي. باستعمال الصبغة المحورة المقاومة للحموضة (صبغة ستامب Stamp stain) لصبغ المسحات المباشرة من العينات النسيجية تمت مشاهدة جراثيم عصوية متناهية في الصغر واشبه بالمكورات مقاومة للحموضة وذات لون احمر براق في 27/68 عينة (39.7%) (مشائم، اجنة، ومسحات مهبلية للنعاج

والماعز). بينما تم عزل جرثومة البروسيلا من 2 (4.54%)، 1 (33.3%%) و(0.0%) من المسحات المهبلية، المشائم ومحتويات معدة الجنين، على التوالي. وقد دلت الفحوصات البيوكيميائية Biochemical والتصنيفية الحيوية Bio-typing على ان العزلات الثلاثة هي النمط الحيوي الاول لجرثومة *Brucella melitensis*. تدل نتائج الدراسة ان جرثومة *Brucella melitensis* ذات النمط الحيوي (1) هي المسبب الرئيسي للاجهاض وانخفاض الخصوبة في المجترات الصغيرة في اقضية محافضة ديالى حيث لم يتم مشاهدة او عزل اية جرثومة اخرى قد تكون مسببة للاجهاض في المناطق التي اجريت الدراسة فيها.

الكلمات المفتاحية: عزل البروسيلا، الاجهاض، النعاج والماعز، ديالى.