Academic International Journal of Veterinary Medicine
ISSN: 2984-7753
Aca. Intl. J. Vet. Med. 2025;3(1) 20-27
Journal homepage: www.aipublishers.org/aijvm

# Evaluation of Diagnostic Tests and Antimicrobial Sensitivity of *Brucella Melitensis* Isolated from Awassi Ewes in Iraq

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(Received 4 January 2025, Revised 17 February 2025, Accepted 1 April 2025, Published 22 April 2025)

## Abstract
This study investigates the prevalence and characteristics of *Brucella melitensis* in unvaccinated Awassi ewes in Iraq. Brucellosis, a zoonotic disease, poses significant challenges in endemic regions due to inadequate vaccination and veterinary knowledge. The research involved serological testing (RBPT, SAT, and MRT) and bacteriological identification on blood and milk samples from 456 ewes, with 452 being clinically healthy and 4 having aborted. Serological tests revealed 30 positive RBPT/SAT results, with varying titers, indicating potential infections. Brucella spp. was isolated from 6 milk samples and a vaginal discharge sample. Biochemical tests identified all isolates as *Brucella melitensis* biotype 3. Antimicrobial susceptibility testing showed sensitivity to Gentamicin, Chloramphenicol, Carbenicillin, and Ampicillin, but varying sensitivity to Cephalothin, Tetracycline, Streptomycin, Kanamycin, and Penicillin, and resistance to Colistin. The study highlights the necessity for local antimicrobial resistance data to inform effective treatment and control strategies, using a One Health approach. The findings suggest integrating serological and bacteriological diagnostics for updated brucellosis management guidelines.

**Keywords**: Brucellosis, *Brucella melitensis*, Zoonotic disease, small ruminants, Awassi sheep

**How to cite:**
Asaad Khalaf Talal Al-Shuwaili, Ali Hussein Fadhil, Evaluation of Diagnostic Tests and Antimicrobial Sensitivity of *Brucella melitensis* Isolated from Awassi Ewes in Iraq *Aca. Intl. J. Vet. Med. 2025;3(1)20-27*: https://doi.org/10.59675/V313U

## Introduction
Brucellosis is a significant zoonotic disease caused by bacteria of the genus *Brucella*, which affects both humans and various animal species, particularly livestock. In small ruminants such as sheep and goats, *Brucella melitensis* is the primary etiological agent responsible for reproductive disorders, including abortion, infertility, and reduced milk production. The condition creates severe problems for

animal health, agricultural production, and public health safety in parts of the world that do not adequately implement animal vaccination protocols and disease control measures.

Brucellosis continues to exist as an endemic health issue throughout Iraq, together with other countries within the Middle East. The disease remains active because livestock farmers follow traditional management practices while experiencing limited access to veterinarians and poor knowledge about the disease. The control of diseases requires prompt detection of infected animals along with their immediate identification. The field surveillance primarily uses three serological tests, which include Rose Bengal Plate Test (RBPT), Standard Tube Agglutination Test (SAT), and Milk Ring Test (MRT), in addition to bacterial isolation and biotyping, which serves as the definitive confirmation method. Brucella strains show antimicrobial resistance, becoming an extra obstacle for treatment and control strategies.

The existing awareness about brucellosis in Iraq fails to match the shortage of contemporary bacteriological data for unvaccinated Awassi sheep herds concerning *Brucella melitensis* strain sensitivity to drugs. Studies mainly depend on serological marks without bacteriological examination or strain identification procedures. Local Brucella strains' antimicrobial sensitivity profiles remain understudied, inhibiting successful brucellosis treatment procedures.

The main goal of this research was to establish Brucella infection rates in unvaccinated Awassi ewes with normal clinical status and those experiencing abortions through serological testing (RBPT, SAT, and MRT). Furthermore, the study aimed to:

- Laboratory personnel will use bacteriological culture and biochemical tests to both detect and identify Brucella organisms from milk and vaginal discharge samples.
- The specific biovar found in the study population will be determined by performing bacteriological characterization of isolated Brucella strains.
- The researchers evaluated how common antibiotics work against isolated pathogens to develop better treatment and control methods.

The infectious disease brucellosis ranks among the most widespread zoonotic diseases worldwide because Brucella melitensis is the primary source of infection in small ruminants [1,20]. The disease leads to major reproductive problems among livestock, therefore representing a serious health concern for people living in endemic areas like the Middle East [2]. The diagnosis of brucellosis uses multiple serological tests, where the Rose Bengal Plate Test (RBPT) and the Standard Tube Agglutination Test (SAT) remain popular because of their sensitivity and easy operation [3]. MRT is an examination method for identifying infected lactating animals by surveilling their milk [4].

Bacterial culture represents the only accepted procedure to identify Brucella species. Identifying species and biovars from bacterial isolates becomes possible through biochemical testing and biotyping methods for disease epidemiology research [5]. Reliance on serological data alone dominates research into Brucella strains obtained from Iraqi sheep, as very few investigations have isolated these strains [6]. The growing worry regarding antimicrobial resistance in Brucella strains demands immediate local sensitivity tests because recent research identified this as an important issue [7].

The research follows the One Health framework that demonstrates the connectedness between animal health with human health, and environmental health. Research on Brucella spp. Prevalence and susceptibility patterns in livestock herds help veterinarians and human doctors control zoonotic disease transmission and treat patients effectively [8]. Integrating serological screening, bacteriological confirmation, and antimicrobial profiling forms a comprehensive surveillance and disease management approach in line with international standards [9].

Multiple investigations in Iraq about brucellosis have primarily focused on serological tests but failed to use bacteriological confirmation [6,10]. Few studies demonstrate data collection on local Brucella isolate biotypes today, and none monitor these bacteria's current antimicrobial resistance patterns. The existing literature fails to provide enough data about how different serological tests match each other regarding their ability to detect infected animals in the field. The absence of such data creates obstacles for developing targeted control programs that match local Brucella epidemiology patterns.

This study combines a thorough analysis of *Brucella melitensis* through serological methods, cultural identification, and antimicrobial testing of unvaccinated Awassi sheep to create updated disease control and treatment guidelines.

## Materials and Methods

### Study Area and Animal Selection

This study was conducted on 456 unvaccinated Awassi ewes from six different flocks in various regions of Iraq. Among these, 452 ewes were clinically normal, while four had experienced abortion. The selected flocks had no history of Brucella vaccination.

### Sample Collection

A total of 456 blood samples and 36 milk samples were collected. Blood samples were obtained aseptically from the jugular vein using sterile test tubes, while milk samples were collected from lactating ewes using sterile containers. One additional vaginal swab was collected from an aborted ewe (animal No. 414).

All samples were transported immediately under chilled conditions to the laboratory. Serum was separated from the blood samples on the same day by centrifugation and stored at 4 °C. Milk samples were stored at 4 °C and processed the following day.

### Serological Testing

All 456 serum samples were subjected to two serological tests:

1. **Rose Bengal Plate Test (RBPT):** Conducted using Rose Bengal-stained *Brucella abortus* antigen (France).
2. **Standard Tube Agglutination Test (SAT):** Performed using *B. abortus* strain 99 antigens (France), following the protocol recommended by the World Health Organization. A SAT titer of ≥40 IU/mL was considered positive.

Out of the total samples:

- 30 sera tested positive for RBPT and SAT (≥40 IU/mL).



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- 4 samples had SAT titers of 31 IU/mL but were RBPT-positive.
- 2 samples with no detectable SAT antibodies still showed positive RBPT results.
- 419 samples were negative in both tests.

The agreement rate between RBPT and SAT in identifying positive cases was 83.33%.

**Milk Ring Test (MRT)**
Milk samples from 36 ewes that tested positive by either RBPT or SAT were further examined using the Milk Ring Test (MRT), which employed a haematoxylin-stained antigen (France).

- Of the 36 samples tested, 25 (69.44%) yielded positive MRT results, indicating the presence of *Brucella*-specific antibodies in the milk.

**Bacterial Isolation and Culture**
A total of 36 milk samples and one vaginal swab were inoculated on selective *Brucella* agar (Tryptose agar enriched with horse serum and antibiotics: Bacitracin 25 IU/mL, Polymyxin B 6.25 IU/mL, Actidione 100 µg/mL). Cultures were incubated at 37 °C for 3–5 days aerobically and in an atmosphere containing 10% $CO_2$.

- *Brucella* spp. was isolated from 6 of the 36 milk samples (16.67%) and the vaginal discharge of ewe No. 414.

**Biochemical Identification**
Colonies were examined macroscopically and microscopically. Isolates were subjected to a series of biochemical tests:

- Hydrogen sulfide production
- **Growth on dye-containing media**: Thionin (1:25,000), basic fuchsin (1:50,000 and 1:100,000), and crystal violet (1:50,000)
- **Urease activity** (positive within 2–4 hours)
- **Slide agglutination test** using monospecific antisera for *B. abortus* and *B. melitensis* (England)

Based on the results—thionin-sensitive, fuchsin- and crystal violet-resistant, and absence of $H_2S$ production—all isolates were identified as *Brucella melitensis* biotype 3.

**Antimicrobial Susceptibility Testing**
All 7 *Brucella* isolates were tested for antimicrobial susceptibility using the disk diffusion method according to standard guidelines. Ten antibiotics were tested:

- Gentamicin (10 µg)
- Chloramphenicol (30 µg)
- Carbenicillin (50 µg)
- Ampicillin (10 µg)
- Cephalothin (30 µg)
- Tetracycline (30 µg)
- Streptomycin (10 µg)

- Kanamycin (30 µg)
- Penicillin (10 µg)
- Colistin (10 µg)

## Results

### Serological Testing

The researchers tested 455 Awassi ewe serum samples with the Rose Bengal Plate Test (RBPT) and the Standard Tube Agglutination Test (SAT). Laboratory tests using SAT revealed thirty samples (6.59%) with positive results when serum titers exceeded 40 IU/mL. The same samples tested positive on RBPT, which correlated well with test results obtained from RBPT.

The Rose Bengal Plate Test showed positive reactions in four serum samples with SAT titres of 31 IU/mL and two serum samples that tested with 0 IU/mL SAT titres. RBPT and SAT produced negative results for the 419 evaluated test samples. 86.67% of shared positive results were detected when measuring the agreement between RBPT and SAT testing methods.

### Milk Ring Test (MRT)

The laboratory evaluated the Milk Ring Test (MRT) on 36 milk samples taken from serologically confirmed ewes. The Milk Ring Test produced positive results for 25 samples among the tested 36 milk samples, which indicates Brucella antibodies exist in milk products.

### Bacteriological Isolation

Six of the 36 (16.67%) milk samples acquired from positive-serology ewes resulted in Brucella organism isolation. The research obtained bacteria from both clinically healthy and aborted animals at three samples each. The bacteriological analysis of vaginal discharge from aborting ewe number 414 produced a Brucella isolate. The laboratory conducted no serological tests because ewe No. 414 lacked available serum and milk samples.

### Among the infected ewes:

The test results of five animals produced positive reactions at every test level for RBPT, SAT and MRT.

The laboratory results indicated that one ewe presented positive results on RBPT and SAT, whereas it showed negative results on MRT.

The vaginal discharge pathology of ewe No. 414 remained the only confirmed method to identify the Brucella isolate because blood and milk testing was unavailable.

Identification and Characterization of Isolates

The bacterial isolates were cultivated successfully on selective media without depending on a $CO_2$ incubation environment. All isolates failed to create hydrogen sulfide within the culture media but reacted to the following dyes:

• Sensitive to thionin at 1:25,000 dilution

• Resistant to thionin and basic fuchsin at 1:50,000 and 1:100,000 dilutions

The strains grew without inhibition from a 1:50,000 crystal violet solution.

Urease activity in the isolates was intense due to the observation of positive reactions within 2 to 4 hours after inoculation. A slide agglutination reaction revealed that the bacteria reacted with Brucella



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abortus and Brucella melitensis antisera. The biochemical features and serological identification showed that all tested isolates were Brucella melitensis biotype 3 strains.

### Antimicrobial Susceptibility Testing

The disc diffusion method was used to evaluate the antimicrobial susceptibility of the seven Brucella melitensis isolates. A comprehensive summary of the obtained data is provided in Table 2. Tests on the Brucella melitensis isolates (n=7) revealed complete sensitivity toward Gentamicin, Chloramphenicol, Carbenicillin, and Ampicillin. Testing with Cephalothin, Tetracycline, Streptomycin, Kanamycin, and Penicillin showed different sensitivity levels between the isolated microorganisms. The study results demonstrated that Colistin failed to exhibit antibacterial effects against isolates.

**Table 1: Relationship Between RBPT and SAT Results in Awassi Ewe Sera**

| No. of Samples Examined | No. of Positive Samples (RBPT) | % RBPT Positive | No. of Positive Samples (SAT ≥40 IU/mL) | % SAT Positive |
|---|---|---|---|---|
| 455 | 36 | 7.91% | 30 | 6.59% |

**Table 2: Antibiotic Susceptibility of *Brucella melitensis* Isolates**

| Antibiotic | Disc Content | No. of Sensitive Isolates | No. of Resistant Isolates |
|---|---|---|---|
| Gentamicin | 10 µg | 7 | 0 |
| Chloramphenicol | 30 µg | 7 | 0 |
| Carbenicillin | 50 µg | 7 | 0 |
| Ampicillin | 10 µg | 7 | 0 |
| Cephalothin | 30 µg | 5 | 2 |
| Tetracycline | 30 µg | 4 | 3 |
| Streptomycin | 10 µg | 3 | 4 |
| Kanamycin | 30 µg | 3 | 4 |
| Penicillin | 10 µg | 2 | 5 |
| Colistin | 10 µg | 0 | 7 |

## Discussion

The findings in this research demonstrate that Brucella melitensis infections exist among unvaccinated Awassi ewes in Iraq, so extensive diagnostic methods and region-specific monitoring become essential for brucellosis control.

### Serological Testing

RBPT and SAT serological tests revealed 83.33% agreement for detecting Brucella-positive animals. Due to its high sensitivity, the RBPT is a vital screening instrument that works best in resource-limited conditions [11]. EBPT positive test outcomes, finding agreement with SAT negative readings, may be explained through the EBPT's ability to generate incorrect positive results in endemic regions [12]. RBPT functions as an effective first-stage screening instrument, but laboratory professionals should perform confirmatory tests using precise assays to achieve accurate diagnosis [13].

### Milk Ring Test (MRT)

The Milk Ring Test correctly identified specific Brucella antibodies in 69.44% of milk samples obtained from Brucella-positive ewes, proving ongoing bacterial secretion. MRT exhibits valuable properties for detecting Brucella antibodies in milk because it helps veterinary staff to identify infected animals in dairy herds, according to research [14]. The sensitivity of MRT must be used with additional diagnostic methods because milk composition and lactation stage affect test results [15].

### Bacteriological Isolation and Biotyping

Brucella organism isolation in research samples from milk specimens (16.67%) and vaginal discharge tissue proves beyond doubt that the animals are infected. According to their biochemical indicators, the isolated bacteria are identified as B. melitensis biotype 3, which matches the predominant minor ruminant strain throughout the Middle East [16]. Effective control methods require specific attention to this biotype identification due to its significance in this region.

### Antimicrobial Susceptibility

Testing results show that Gentamicin, Chloramphenicol, Carbenicillin, and Ampicillin are effective antimicrobial agents for treatment. Resistant patterns were observed regardless of variable and complete potency against Tetracycline, Streptomycin, Penicillin, and Colistin. A systematic review showed that tetracycline-resistant Brucella isolates have a low incidence yet are on the rise, thus suggesting that continuous antimicrobial resistance monitoring should persist [17]. Doctors need to use antibiotics sparingly because aminoglycoside resistance is increasing, although it remains low [18].

### Asymptomatic Carriers

The discovery of Brucella bacteria in healthy ewes proves how asymptomatic carriers function as disease transmission agents. Such animals maintain their health status while carrying the pathogen, thus making the control of disease transmission more challenging. Regular screening through both serological and bacteriological tests plays an essential role in detecting and properly handling pathogen carriers [19].

## Conclusion

Accurate diagnosis of Brucella infections requires combining diagnostic tests because the findings are essential in detecting these infections. RBPT functions as a first diagnostic screening tool, but SAT and MRT tests better confirm the diagnosis of brucellosis. Continuous surveillance and proper antimicrobial prescription practices have become essential because antibiotic-resistant strains have appeared. The control programs for brucellosis in endemic areas must focus on resolving the problem of asymptomatic carriers to be effective.

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