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Research article

Seroprevalence and molecular diagnosis of sheep brucellosis in Dakahlia governorate, Egypt

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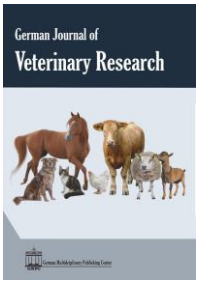

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Abstract

Brucellosis is an endemic disease among livestock and humans in Egypt. Sheep are the most common type of livestock ruminant in Egypt and are considered the fundamental etiology for spreading and maintaining *B. melitensis* either in human beings or animal populations. In the current study, we investigated the seroprevalence of brucellosis in sheep herds reared in Bilqase, one of the biggest cities at Dakahlia governorate in Egypt's Delta region. In total, 610 sheep from seven herds were investigated. Anti-*Brucella* antibodies were detected in 48 (7.8%) samples tested by Buffered Acidified Plate Antigen Test (BAPAT), in 44 (7.2%) samples tested by Rose Bengal Plate Test (RBPT), and in 41 (6.7%) samples tested by Milk Ring Test (MRT). The isolation rate was 29.6% (16 out of 54 examined samples). *Brucella* organism was isolated from three aborted fetuses, one tissue sample of slaughtered serologically positive ewe, and 12 milk samples. The Abortus Melitensis Ovis Suis-PCR (AMOS-PCR) confirmed all *Brucella* strains as *B. melitensis*. More than three successive negative serological tests are required to declare that the infected herd is free from brucellosis. In conclusion, no single serological test could conclusively diagnose brucellosis in endemic areas. Confirmation of results with molecular diagnosis or culture is indispensable in diagnosis. *B. melitensis* was the prevalent serotype among sheep in Dakahlia governorate.

Keywords: Brucellosis, Sheep, Milk, Serology, Isolation, Egypt

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Introduction

Brucellosis is a ubiquitous zoonotic disease and endemic in Egypt for thousands of years. Brucellosis has been detected at high levels among ruminants nationwide, particularly in large intensive breeding farms (Refai, 2002; Wareth et al., 2014). Sheep are the most common and widespread livestock species in Egypt due to their ability to graze on a large scale and their need for little care (FAOSTAT). On the other hand, the number of cattle herds has increased because of their greater utilization for meat and milk production (compared with buffaloes). The current number of sheep in the Egyptian field is 5.69 million heads. The majority of these animals are reared in the open system, such as mobile grazing flocks in the desert, Bedouin areas, and green fields between villages and towns (Elshazly and Youngs, 2019). Individuals' transmission of brucellosis occurs through close contact with contaminated abortion discharges and fetal membranes or commonly through the consumption of infected non-pasteurized milk and dairy products (van den Brom et al., 2020).

In general, the diagnosis of brucellosis in sheep is challenging and is mainly based on bacteriological and immunological tests (Ren and Peng, 2020). *Brucella* is excreted in huge quantities at parturition and could be cultured from a broad spectrum of materials such as vaginal discharges, placenta, fetal stomach contents, and milk using suitable selective culture media (Ebrahimi et al., 2014). The fecal and environmental contamination with the infected material is maintained to a minimum to give the highest possibility of an effective isolation rate. However, if additional material is not available or contaminated, the fetal stomach contents are commonly otherwise sterile and a good source for *Brucella* isolation. Suitable materials for isolation also include supra mammary, internal iliac, and retropharyngeal lymph nodes, udder tissues, testes, and gravid uterus. Bacterial colonies might be provisionally recognized as *Brucella* based on their colony morphology and appearance. However, the conclusive identification of *Brucella* spp. can only be achieved using specific procedures described at *Brucella* reference centers (El-Diasty, 2009).

In Egypt, sheep are the main source for spreading and maintaining *B. melitensis* in humans and animals (El-Diasty, 2009).

Indeed, the rearing system of sheep in Egypt as a migratory flock and the easy way to migrate between different territories represents a smooth way to transmit the infection (Abdel-Hamid et al., 2017). The clinical manifestations of brucellosis in sheep are reproductive disorders, abortion, retained placenta, orchitis, and epididymitis (OIE, 2016). Routine diagnosis of brucellosis and comprehensive surveys on the prevalence of ovine brucellosis, especially in developing countries, are still exclusively performed by serological tests (screening and confirmatory) due to their low price, easily applied and highly sensitive (Ducrotoy et al., 2018; Kalleshamurthy et al., 2018).

Diagnosis based on serology, slaughtering of seropositive sheep, accompanied by vaccination of negatives, and applying strict hygienic measures constitutes the practical eradication program for control of bovine brucellosis in endemic areas (Hashem et al., 2020). It is worth mentioning that *B. melitensis* biovar 3 was and still is the predominant pathovar in sheep in Egypt (Refai, 2002; Wareth et al., 2014; Abdel-Hamid et al., 2020; Hegazy et al., 2020; Wareth et al., 2020). Molecular techniques such as Polymerase Chain Reaction (PCR) are the most efficient method for detecting *Brucella* spp. from bacterial isolates, as it is an accurate technique that allows the rapid diagnosis of brucellosis (Fekete et al., 1990; Baddour, 2012). Thus, the current study aimed to estimate the herd prevalence of brucellosis in sheep in a city of Delta region by serology and identification of circulating *Brucella* species by conventional bacteriological methods and PCR. Furthermore, the test and slaughter strategy was evaluated in two herds.

Materials and methods

Sampling

A total of 610 sheep belonging to seven herds and reared as mobilized flocks moved between different villages were investigated for brucellosis incidence (Table 1). The evaluation of the test and slaughter program was performed on herd no.2 (120 animals) and herd no.4 (115 animals) because these two herds were under our continuous supervision, and communication with the sheep holders was easier. Animals were examined every three weeks, and positive animals were removed immediately for slaughtering. Animals were serologically tested until three successive negative tests were obtained. A total of 41 milk samples were taken from seropositive ewes. Stomach content, spleen, liver, and lung specimens were taken from three aborted fetuses, and tissue specimens (liver, spleen, and lymph nodes) from ten seropositive ewes were collected after slaughtering for isolation.

Serology

All serum samples were subjected to the Rose Bengal Plate Test (RBPT) and Buffered Acidified Plate Antigen Test (BAPAT) as screening tests according to the OIE Manual (OIE, 2018). Antigens were obtained from the Veterinary Serum and Vaccine Research Institute, Abbassia, Cairo, Egypt. Milk samples were examined using the Milk Ring Test (MRT) obtained from AHVLA, New Haw, Addlestone, Surrey KT15 3NB, UK. MRT was carried out according to the OIE Manual (OIE, 2016).

Bacteriological isolation and identification

Isolation of *Brucella* species from milk samples, aborted foeti, and tissue specimens was carried out according to the FAO/WHO Expert Committee's recommendations on Brucellosis (Alton et al., 1988; OIE, 2016). Briefly, all suspect colonies were identified as *Brucella* spp. using classical biotyping methods according to colony morphology, biochemical tests (oxidase, catalase, urease), CO_2 requirement, H_2S production, growth in the presence of thionin and fuchsine dyes, reaction with mono-specific anti-sera (A, M, R) and agglutination with trypaflavine and crystal-violet. *Brucella* isolates were further molecularly characterized at the species level using Abortus Melitensis Ovis Suis-PCR (AMOS-PCR) as previously described by (Scholz et al., 2008; Matope et al., 2009). Briefly, 25 µl of a reaction mixture containing 10× PCR buffer, 10mM of deoxynucleotide triphosphates (dNTPs), and 10 pmol/µl of primers (0.2 µM each) of *B. abortus*, *B. melitensis*, *B. ovis*, *B. suis,* and IS711-specific primer, 0.2 µl of 5U/µl of Taq DNA polymerase was used. HPLC was used to complete the 25µL. A total of 1µl DNA extraction template was added to the 24µL reaction mixture. The PCR was performed with Thermocycler. Amplification was performed with an initial temperature of 95°C for 5 minutes. This was followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 58°C for 2 minutes, and elongation at 72°C for 2 minutes. The PCR products were incubated for a further five minutes at 72°C to allow elongation of products before storage at 4°C. The PCR products were separated by electrophoresis using 1.5% agarose gel (w/v). Visible bands were considered positive reactions of appropriate sizes of (498 bp) for *B. abortus*, (731 bp) for *B. melitensis*, (976 bp) for *B. ovis*, and (285 bp) for *B. suis*.

Results and discussion

Brucellosis is considered one of the serious zoonotic diseases affecting animals and humans, caused by the genus *Brucella*. The significance of *B. melitensis* for sheep and goats changes with the geographic locality and can be affected by husbandry practices and the susceptibility of sheep breeds in the region. Thus, the present study intended to investigate the prevalence of brucellosis in sheep herds by commonly used diagnostic serological, bacteriological, and molecular procedures and to identify the causative *Brucella* species in the study area.

Serum and milk samples of 610 sheep belonging to seven herds from Mahfoza village at Bilqase town of Dakahlia governorate were examined for brucellosis between November 2016 and April 2019. Different serological tests, including BAPAT, RBPT, and MRT, were employed. As shown in Table 1, 7.8% (48/610), 7.2% (44/610), and 6.7% (41/610) of animals were positive for BAPAT, RBPT, and MRT, respectively.

Table 1: Seroprevalence of brucellosis in seven different sheep herds using BAPA, RBPT, and MRT at Dakahlia governorate

Herd	Number of animals	Test [1]		
		BAPAT no (%)	RBPT no (%)	MRT no (%)
Herd 1	70	3 (4.2)	3 (4.2)	3 (4.2)
Herd 2	120	7 (5.8)	5 (4.1)	5 (4.1)
Herd 3	109	9 (8.2)	9 (8.2)	7 (6.4)
Herd 4	115	12 (10.4)	11 (9.5)	11 (9.5)
Herd 5	93	8 (8.6)	8 (8.6)	8 (8.6)
Herd 6	63	4 (6.3)	3 (4.7)	3 (4.7)
Herd 7	40	5 (12.5)	5 (12.5)	4 (10)
Total	610	48 (7.8)	44 (7.2)	41 (6.7)

[1] Abbreviation: BAPAT; Buffered Acidified Plate Antigen Test, RBPT; Rose Bengal Plate Test, MRT; Milk Ring Test.

Table 2: Number of *Brucella* isolates obtained from aborted fetuses, milk, and tissue samples

Animal	Aborted foeti		Milk samples		Tissue specimens		Total samples	Total positive
	Samples No.	Isolates No.	Samples No.	Isolates No.	Samples No.	Isolates No.		
Positive ewes	3	3 (100%)	41	12 (29.2%)	10	1 (10%)	54	16 (29.6%)

The difference in seropositivity may be attributed to differences in sensitivity and specificity of the employed serological tests. BAPAT is more sensitive as a screening test and can complement RBPT in the program of control of bovine brucellosis. However, RBPT is more specific than BAPAT due to its pH, which inhibits the non-specific agglutinins (Corbel, 1973). MRT diagnosed fewer positive cases than BAPAT and RBPT because MRT may be less sensitive to detect antibodies in milk containing low concentrations of antibodies or due to fat clustering factors (OIE, 2000). Brucellosis is a common zoonosis in Egypt, and the disease is prevalent nationwide among cattle, buffaloes, sheep, and goats (Wareth et al., 2014). Surveillance and control of brucellosis in small ruminants can be improved by decreasing the proportion of uncontrolled movement of animals between villages, preventing trade in open markets, and regular performance of serological diagnosis and application of mass vaccination (Hegazy et al., 2020). Isolation and identification of *Brucella* is still the gold standard for diagnosis and is considered an important tool to assure the flock's status and support the serological findings. In the current study, an inflation rate of 29.6% was observed. Sixteen *Brucella* strains were isolated from all examined aborted fetuses (3/3), from one tissue specimen (1/10), and from milk samples (12/41) (Table 2). The low isolation rate (10%) obtained from tissue specimens of seropositive ewes may be attributed to the possibility of contamination of the samples and the fastidious nature of *Brucella* organisms. Smears from *Brucella* cultures were stained by the modified Ziel-Neelsen stain. Stained smears showed the presence of large aggregates of weakly acid-fast organisms, which is considered presumptive evidence of *Brucella* infection. They are not truly acid-fast but are resistant to decolonization by weak acids and thus stain red by modified Zeil-Nielsen's method (OIE, 2012). All isolates presented smooth, transparent, and convex colonies with intact borders. The surface was brilliant and gave a honey color under transmitted light.

AMOS-PCR was an effective method for rapid, sensitive, and accurate *Brucella* identification at the species level. AMOS-PCR identified all isolates as *B. melitensis*, the predominant *Brucella* spp. circulating in humans and livestock in the Middle East and Mediterranean countries, including Egypt (Abedi et al., 2020; Al-Sherida et al., 2020; Ebid et al., 2020; Wareth et al., 2020). In Egypt, *B. melitensis* has been isolated from cattle, buffalo, sheep, goats, camels, and humans (Abdel-Hamid et al., 2020; Sayour et al., 2020; Wareth et al., 2020). PCR technique is a sensitive and specific technique for direct detection of *Brucella* in serum samples from sheep and goats (Wareth et al., 2015) and milk samples (Abdali et al., 2020). BAPAT and MRT results were used to evaluate the efficacy of test and slaughter strategy in herd no. 2 and herd no. 4, respectively (Table 3). The results showed that the periodic testing of sheep with rapid elimination of positive cases could virtually eliminate the source of infection among sheep through the slaughtering of positive reactors, resulting in a gradual decrease

Table 3: Evaluation of test and slaughter strategy in two sheep herds depends on the results of BAPAT and MRT results

	BAPAT[1] results Herd number 2			MRT[2] results Herd number 4		
Test	No. of animals	Positive cases	Negative cases	No. of animals	Positive cases	Negative cases
1st	120	7	113	115	11	104
2nd	113	4	109	104	8	96
3rd	109	3	106	96	5	91
4th	106	3	103	91	5	86
5th	103	0	0	86	6	80
6th	103	0	0	80	2	78
7th	103	1	102	78	1	77
8th	102	0	102	77	1	76
9th	102	0	102	76	0	76
10th	102	0	102	76	1	75
11th	102	0	102	75	0	75
12th	102	0	102	75	1	74

[1] BAPAT; Buffered Acidified Plate Antigen Test [2] MRT; Milk Ring Test.

of prevalence to zero, beginning from the 8th examination up to the 12th examination using BAPAT. On the other hand, based on MRT results obtained in herd no.4, it was shown that MRT compromised the identification of positive animals and failed to diagnose the infection inside the herd due to its inability to detect the low antibody concentration in milk, especially after parturition and abortion.

Using more than one serological test to diagnose positive cases is indispensable to eradicate the infection from the herds in endemic areas. The early release of the herd out of quarantine should be avoided, especially under unhygienic conditions and a lack of controlled animal movement (Hosein et al., 2018). Test and slaughter programs are useful for managing outbreaks, especially when the high number of animals makes the implementation of stamping-out unfeasible. However, the combination of test and slaughter programs with mass vaccination will be a better eradication strategy. The appearance of positive cases after several successive negative results may be explained by the presence of other sources of infection in the herd, such as carriers, e.g., dogs and cats (Wareth et al., 2017), the presence of latent infection (El-Diasty et al., 2018) or due to survival of the organisms in the pasture for an extended period.

Conclusions

In conclusion, this study highlights the predominance of *B. melitensis* in Egypt and its potential risk for humans and animals. No single test was capable of conclusively diagnosing brucellosis in endemic areas. *B. melitensis* is the prevalent species among sheep in the Dakahlia governorate. Unhygienic conditions, a husbandry system favoring mixed populations of different ages and sexes, and environmental contamination make the diagnosis of brucellosis challenging and difficult to eradicate. Thus, serology has to be combined with molecular diagnosis to accurately identify positive cases, and test and slaughter must be accompanied by vaccination.

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