Comparative study on serological, bacteriological, and molecular methods for diagnosis of Brucellosis in the milk of cows with reproductive disorders

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Abstract

Background

Brucellosis is a significant animal disease communicable to humans that affects cattle worldwide. It is spreading disease in many developing countries. Brucellosis in farm animals induces serious economic costs in undeveloped countries and causes serious health hazards to dairy users. Sensitive and accurate diagnostic techniques are necessary for disease surveillance and management.

Findings

In this investigation, the overall prevalence of brucellosis in examined animals was 34.91% by MRT, while i-ELISA showed 33.96% prevalence. the sensitivity and specificity of MRT were 93.55%, and 89.33% and ELISA were 96.77%, 92 % respectively. whereas, the false positive was 10.67% and 8.00% for MRT and ELISA respectively. *Brucella* microorganism was isolated in 25.47 % milk samples, all *Brucella* strains were determined as *B. melitensis* biovar 3.

By conventional PCR and real-time PCR, 28.3% of samples were positive. The results showed that the sensitivity of bacteriological culture, Conventional PCR, and Real-time PCR was 87.10%,96.77%, 96.77% respectively while specificity was 100% each, and estimated false positive was zero% each.

Conclusion

The milk ELISA had detected high sensitivity and specificity than those of the milk ring test. *B. melitensis* biovar 3 is the predominant strain isolated in the milk of cows with reproductive disorders. The PCR's high sensitivity and specificity, as well as its speed, and low risk to laboratory workers, make it an extremely useful technique for brucellosis diagnosis. The possibility of contaminated raw milk and raw dairy products has surfaced, providing a serious public health threat.

Background

Brucellosis is an old disease that the Romans was reported more than 2000 years ago.

Brucella melitensis was isolated by Bruce in 1887[1].

Brucellosis is a transmissible disease that has a severe economic impact on livestock. The disease is caused by bacterial members of the Brucella family, which infect only certain animal species. Most Brucella species, however, can also infect other animals. Brucellosis is a highly contagious human zoonosis. Consuming fresh milk from affected animals is the most prevalent way for humans to become affected. As a consequence, people suffer from a severe crippling sickness. Veterinarians, farmers, and abattoir employees are at risk of becoming infected because they work with infected animals.

Animals acquire brucellosis through direct contact with diseased animals during community herding and grazing or indirect by contact with Infected animals' biological secretions, including sexual secretions, milk,

aerosols, and abortive materials containing bacteria [2].

Brucellosis becomes a health threat when bacteria from diseased animals infect humans through the food of animal origin. the main challenge to brucellosis eradication is the accurate diagnosis. While numerous serological techniques for disease detection in cattle, such as the Rose Bengal tube test, serum agglutination test, and enzyme-linked immunosorbent assay (ELISA), are performed, they are often seen to be deceptive. Recently, confirmation based on PCR, in association with serological tests, has been popular for ensuring accurate diagnosis [3]. It poses financial concerns for cattle producers or product consumers.

this study aimed to the comparison between different techniques (serological, bacteriological, and molecular) for the diagnosis of Brucellosis in milk methods of cows with reproductive disorders.

Material And Methods

106 milk samples were obtained from cows with records of reproductive abnormalities (n=74) from various locations, including late aborted animals (n=22), cows with a record of retained placenta (n=28), repeat breeders (n=24), and seemingly healthy cows (n=32).

Milk samples were collected a non-invasive collection of milk samples by traditional hand-stripping was approved by the Egyptian Network of Research Ethics Committees (ENREC) and under the supervision of authors. Fifteen ml of the milk were sampled and subdivided into 5 ml aliquots. These were used in the serological, bacteriological, and molecular examination. Samples collected for PCR assay were kept at –80 °C until examination.

Serological examination of milk Samples

Milk Ring test (MRT)

30 µ1MRT antigen (Animal Health Veterinary Laboratories Agency (AHVLA), DEFRA, UK) was mixed with 1ml of fresh milk samples (within 2 hours after collection) mixed well, and kept at 37 °C for 3 hours. The appearance of the purple band at the top of milk was considered positive [4].

Indirect Enzyme-Linked Immunosorbent Assay (i-ELISA)

The anti-brucella antibodies were detected using an indirect ELISA (i-ELISA) kit (Svanova, Sweden), and the technique was conducted according to the manufacturer's instructions. Before usage, milk samples were centrifuged at 5000 rpm for 5 minutes to remove the cream layer. Using an ELISA reader, the control and test-sample wells' optical densities (OD) were adjusted at 405nm wavelength (ThermoElectron, Finland). The results were then interpreted according to the manufacturer's instructions.

Bacteriological examination of milk Samples

Milk samples were inoculated onto Brucella agar (Oxoid) plates supplemented with Brucella supplement (Oxoid) and 5% horse serum. The plates were then incubated at 37°C in 10% CO2 for 5 to 10 days and

examined daily for the presence of colonies. The obtained colonies were identified and classified according to Quinn et al. [5].

Molecular examination of milk Samples:

DNA extraction from milk samples

DNA was extracted using NZY tissue g DNA isolation kit® according to the manual. 1400 ul of milk samples were transferred to 1.5 microcentrifuge tubes and centrifuged at 3000 rpm for 5 minutes. Then discard the supernatant (milk and cream) and pellets were transferred into new microtubes. Finally, extracted DNA was stored at -80 ºC until used.

Conventional PCR

For conventional PCR, pair of oligonucleotide primers **B4: TGG CTC GGT TGC CAA TAT CAA-3,** and **B5: CGC GCT TGC CTT TCA GGT CTG** were used and amplified a 223-bp fragment [6] using 2X Taq PCR mix (Tiangene Cat. no. KT201)

The optimized cycle program for PCR was as follows: at 94 ◦C for 3 min., afterthose 40 cycles of the 20s at 95◦C, 1 min at 60 ◦C, and 1 min at 72^{o}C. The last cycle included incubation of the sample at 72 ^{o}C for 7 min. The PCR was performed in a DNA thermocycler (Perken Elmer model 9600). The negative control contained sterile water instead of the DNA template, while, the positive control was *B. melitensis* Rev1DNA.

Electrophoresis of PCR product:

After amplification, 5ul of the PCR product was mixed with 1ul of 6X gel loading dye and subjected to electrophoresis on 1.5% agarose gel at 100V for 30 min. The gel was stained with ethidium bromide and photographed on a UV transilluminator.

Samples were considered positive for *Brucella* species by using B4 and B5 primers when a single band of DNA at 223 bp was evident in the ethidium bromide-stained gels, compared with the molecular size marker (50 bp DNA ladder).

DNA amplification in Real-Time PCR:

Extracted DNA was amplified by using a real-time PCR detection kit for Brucella (Primer design, JN68G10-21593). The reaction mixture was composed of 5 ul of DNA template and 10 ul of 2x Precision ^{TM} master mix in a tube, 1 ul of primer/probe mix, and RNAse/DNAse free water till reached 20 ul. The optimized cycle program was as follows: 1 cycle 37^{o}C for 15 min. for UNG (Uracil-N-Glycosylase) treatment: initial denaturation at 95^{o}C for 10 min., then 50 cycles consisting of 95^{o}C for 10 s and 60^{o}C for 1 min. To confirm the absence of contamination and false-positive result, a negative and positive control reaction respectively were included in the reaction.

Estimation of Relative Sensitivity, specificity, and estimated false positives of different diagnostic tests:

In this study, the relative sensitivity, specificity, and estimated false positive of different serological tests employed in this study were calculated according to Parikh et al., [7].

Relative Sensitivity= *True positive / True positive + false negative x 100*

Relative Specificity= *True negative / True negative + false positive x 100*

Estimated false positive**=** False positive/ False positive **+** True negative *x 100*

Where: True positive or negative reactions are those confirmed as being positive or negative by other two or more tests. False-positive or negative reactions are those confirmed as being positive or negative by other one or non-tests.

Results

Results of Serological examination

The overall prevalence of brucellosis in examined animals was 34.91% (n=37) by MRT, while i-ELISA showed 33.96% (n= 36) positive (Tables 1 and 2). In the present study, the MRT had detected more positive cases than milk ELISA**.**

Results of Bacteriological examination

In this study, *Brucella* microorganism was isolated in 25.47 % **(**n**=**27) milk samples (Tables1 and 2), all *Brucella* strains were determined as *B. melitensis* biovar 3.

Results of Molecular examination

Out of 106 milk samples, 30 samples (28.3%) were positive by conventional and real-time PCR each (Tables 1 and 2).

Table 1. Serological, bacteriological, and molecular results

Reproductive status	n= of sample	MRT		ELISA		Bacteriological culture		Conventional PCR		Real-time PCR	
		n=	%	n=	%	n=	%	n=	%	n=	%
Late abortion,	22	19	86.36	19	86.36	17	77.27	18	81.81	18	81.81
Retained placenta	28	9	32.14	9	32.14	7	25.00	9	32.14	9	32.14
Repeat breeder	24	7	29.17	6	25.00	3	12.5	3	12.5	3	12.5
Apparently healthy	32	2	6.25	2	6.25	0	0	0	0	0	0
Total	106	37	34.91	36	33.96	27	25.47	30	28.3	30	28.3

Table (2) Agreement between the results of different tests

n= of examined animals	n= of cases	MRT	ELISA	Bacteriological culture	Conventional PCR	Real-time PCR
106	24	+	+	+	+	+
	1	-	-	+	+	+
	5	+	-	-	-	-
	4	+	+	-	+	+
	1	-	+	+	+	+
	4	-	+	-	-	-
	2	+	+	-	-	-
	1	+	+	+	-	-
	1	+	-	-	-	-
	63	-	-	-	-	-

Results of Relative Sensitivity, specificity, and estimated false positives of different Screening test

As shown in Table 3. the sensitivity and specificity of MRT were 93.55%, and 89.33% respectively, whereas, the false positive was 10.67%.

Relative sensitivity, relative specificity, and estimated false positive of indirect ELISA were 96.77%, 92.00%, and 8.00% respectively. The milk ELISA test had high sensitivity and specificity than the milk ring test.

Bacteriological culture, Conventional PCR and Real-time PCR their sensitivity was 87.10%,96.77%, 96.77% respectively while specificity was 100% each, and estimated false positive was zero% each.

Table (3) Relative sensitivity, Relative specificity, and estimated false-positive percentage of different tests:

	Serological tests		Bacteriological tests	Molecular tests	
	MRT	ELISA	Bacteriological culture	Conventional PCR	Real-time PCR
Relative sensitivity	93.55	96.77	87.10	96.77	96.77
Relative specificity	89.33	92.00	100	100	100
Estimated false positive	10.67	8.00	0	0	0

Discussion

Brucella antibodies in milk are commonly detected by serological tests such as MRT and ELISA [8]. MRT is the most extensively performed test for brucellosis checking and detection in dairy cow [9].

In this investigation, MRT detected more positive instances than the milk ELISA. MRT revealed a 34.9% overall prevalence of brucellosis in tested animals, while i-ELISA revealed a 33.96% prevalence (Table 1). These findings support the findings of [10], who mentioned that the MRT gave more positive results 55.6% than the ELISA 29.6%.

In this investigation, MRT showed 93.55%, Relative sensitivity, 89.33% specificity, and 10.67% false positive. Although, Relative sensitivity, relative specificity, and estimated false positive of iELISA were 96.77%, 92.00%, 8.00% respectively, (Table 3). The milk ELISA had detected high sensitivity and specificity than those of the milk ring test. This data confirms previous research by Chisi et al [11] that found milk ELISA to be much more sensitive than MRT.

Variations in sensitivity and specificity of serological tests are caused by many factors such as the animal's sample infection status to be unknown, as well the sample's transport and storage conditions, sample's collection to the laboratory, and also, aspects that the owners have not given (i.e. vaccination status). Even if an infectious status based on culture is available, the finding is still influenced by unknown variables [11].

A high positive MRT result might be caused by numerous milk cases such as mastitis, colostrum, or milk toward the end of the lactation cycle [12].

True negative PCR results would directly correlate with false-positive ELISA responses to cross-reactions with the Lipopolysaccharide of several other bacteria (e.g., *Yersinia enterocolitica* O:9). Although *Yersinia enterocolitica* is common in milk-producing flocks around the world, its distribution in Egyptian flocks remains unclear [13].

Brucella spp. is a fastidious bacteria that need a lot of nutrients to grow. Furthermore, it needs a significant number of viable bacteria in clinical samples, adequate storage, and prompt transport to the diagnostic

laboratory for isolation [14]. In this investigation, *Brucella* spp. was 25.47%, all *Brucella* strains were determined as *B. melitensis* biovar 3. This finding suggests that *B. melitensis* biovar 3 is the most common isolated strain in the milk of cows with reproductive disorders. This finding is in line with the observations of El-Nesser et al [15] and Mitrov et al [16], who noted that the increased proportion of Brucella melitensis might be associated with keeping cattle in near vicinity to sheep and goats, where brucella is generally transferred from animal to animal through contact after abortion. Cattle infection with *B. melitensis* of special importance, due to *B. abortus* does not adequately protect against *B. melitensis* infection [17]. In the present investigation, there were no false-positive samples in the bacteriological culture method. This study supports the findings of da Silva Mol et al [18] who mentioned that Brucella isolation by culture method is the "gold standard." The fact that these samples were collected from different animals at different stages of infection and some of the animals may not be in the active state of shedding organisms in their milk may explain the lower sensitivity and higher specificity (87.10% and 100%, respectively) achieved in the current study [19].

Genetic identification of brucellosis using PCR methods is becoming more popular as a standard method. PCR techniques at the genus level are low-cost screening techniques with the potential to detect low DNA levels [20]. Real-time PCR and conventional PCR revealed that 28.3% of 106 milk samples were positive. this result of molecular methods is lower than that found by ELISA. These findings are in line with those of Ducrotoy et al [21] who explained that the failure of PCR in ELISA positive milk samples may be due to irrespective of systemic bacteria or DNA, antibody titers stay elevated for a long time after infection or animals in the chronic phase of the disease.

Milk includes large concentrations of Ca2+, proteinase, fat, polysaccharides, and milk proteins, all of which hinder DNA amplification by PCR reaction. Extraction of purified DNA from the milk sample is crucial in the performance of PCR experiments [22]. To enhance the simplicity and convenience of the present study we used tubes. DNA was picked up using NZY tissue g DNA extraction kit and DNA amplification using primer design real-time PCR kits.

The obtained data showed that the employed assays could be used for risk analysis investigation during the routine control of milk, especially as they were able to detect Brucella DNA in ELISA-negative samples.

Correlation between the rates of Brucella infection with the reproductive status of the examined animals (Table 1) revealed that the rate of Brucella infection was higher among cattle with a history of reproductive disorders than those without a history of reproductive disorders. These findings are harmonious with the results recorded by Tasiame et al [23] who reported that abortion, retained placenta, and infertility were correlated to infection with brucellosis. On the other hand, animals that were seropositive and had no history of reproductive disorders constitute the major sources of infection and control failure.

The obtained data (Table 1) revealed that brucellosis was higher in aborted cows than in cows with retained placenta and repeat breeders, depending on the kind of reproductive problems. Based on the findings, we can conclude that Brucella infection is still greater among cattle with a history of reproductive problems, necessitating additional efforts and effective disease control and eradication plan.

In endemic countries, Brucella infection spreads through infected milk and dairy products, presenting a growing threat to people and entire families [24). In most areas, the trade of non-pasteurized milk and raw milk products should be strictly controlled and limited to certified Brucella-free farms. Local farmers and consumers must receive basic health education about the disease's nature and ways of transmission through milk products. In addition, in light of current scientific findings, a longstanding idea that raw milk is superior to pasteurized milk must be addressed. In locations where the disease is endemic, culture tests are insufficiently sensitive, and serological testing is not highly specific. The amplification of certain segments of DNA has been established in this work. This study has shown that PCR amplification of certain brucella DNA sequences is a far more sensitive technique than culture and that their positive for diagnosis is quite specific.

Conclusion

In conclusion, the PCR's high sensitivity and specificity, together with its speed, variety in sample handling, and reduced risk to laboratory employees, make it a very valuable tool for brucellosis diagnosis. The intalk of possibly contaminated raw milk and raw dairy products poses a severe public health concern. To avoid infection or the spread of infections, general health education on the nature of the disease and the modes of transmission through milk products is often essential.

Declarations

Ethics

This study was carried out in strict accordance with the Guidelines of the Egyptian Network of Research Ethics Committees (ENREC), which complies with the international laws and regulations regarding ethical considerations in research. All efforts were made to minimize animal suffering.

Consent for publication

I, the undersigned, give my consent for the publication of identifiable details, which can include details within the text (“Material”) to be published in the above Journal and Article.

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Competing of Interest

None of the authors has any Conflicts of Interest.

Availability of data and materials

All data generated or analyzed during this study are included in this published article

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Authors' Contributions

Nashwa M. Helmy: data curation, writing editing, Hoda M. Zaki: data curation; Aalaa S. Saad: writing. All authors reviewed the manuscript.

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