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Detection of *Brucella* infection in dairy buffaloes using conventional and molecular methods

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ABSTRACT

This study was conducted on different dairy buffalo herds. A total of 316 buffaloes including 290 buffaloes suffering from reproductive disorders and 26 apparently healthy buffaloes in different rural areas belonging to the Al-Qalyubia Governorate were employed in this study. Epidemiological data revealed that these animals had no history of vaccination against Brucellosis. Blood sera of these animals were subjected to serological assessments using the Rose Bengal plate test (RBPT), Competitive Enzyme Linked Immunosorbent Assay (cELISA), complement fixation test (CFT) in addition to the use of real-time PCR (rtPCR). MRT and bacteriological examination were performed on milk samples. The obtained results showed that the overall sero-prevalences of brucellosis were recorded as 17.41% , 17.09 % , 16.77% , and 17.09% by RBT, c-ELISA, CFT and rtPCR respectively. *B. melitensis biovar 3* was isolated from milk samples 47 (14.87%) It was concluded that for the diagnosis of brucellosis in dairy buffaloes, MRT and RBT remain recommended for screening, CFT is advised for confirming infection in individual animals, and cELISA can be used instead of CFT in some cases. Using rtPCR on serum samples should be regarded as an additional diagnostic technique for detecting and identifying *Brucella* infection and should be considered in conjunction with other serological tests that can progress and conquer the limits of different immunoassays. According to bacteriological testing, *B. melitensis* biovar 3 is still the prevalent *Brucella* field strain found in the milk of buffaloes suffering from reproductive problems.

INTRODUCTION

Brucellosis is a highly contagious, zoonotic disease of farm animals and humans, where it has been frequently recorded in cattle, buffaloes, sheep, goats and camels, as well as dogs as carrier hosts **(Wareth et al. 2017)**.

Buffalo is the main dairy animal of the majority of smallholder farmers in many develop-

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ing countries **(Perera 2008)**. In buffaloes, brucellosis is one of the most common reproductive diseases capable of causing abortion throughout the reproduction season in the last third of gestation. Retention of the fetal membranes, stillbirths and decrease in milk yield result in significant financial losses **(Neta et al. 2010, Dadar and Alamian, 2020).**

The shedding of *Brucella* spp. in milk is posing a growing public health concern to Egyptian consumers. Consumption of dairy products made from unpasteurized milk obtained from small-scale farms where the farmers are working in unsanitary conditions poses an unacceptable risk to public health **(Wareth et al. 2014, Dadar et al. 2021)**.

The most common methods for diagnosing of brucellosis are serological and bacteriological isolation **(Refai 2003)**. The immunoassays for brucellosis in bovines are usually carried out as a part of the disease eradication and surveillance program. In dairy herds, the main methods for testing for brucellosis in domestic animals include the *Brucella* milk ring test and different blood serological tests as reported by **Glynn and Lynn (2008).** Serological assays are used as quick and less expensive diagnostic tools. However, serological tests have many limitations concerning specificity and sensitivity, particularly in animals **(Kaltungo et al. 2014)**. There is a requirement for information related to the comparative serological diagnosis of brucellosis in buffaloes, and it is likely that different animal species may react in different ways to the infectious agent owing to the genetic unpredictability of individual animals, resulting in different test results. Although, a variety of serological tests exist, no lone test is appropriate in all epizootiological studies owing to the disparity in their sensitivity and specificity **(OIE 2019).**

The gold standard for the diagnosis of brucellosis is the isolation of the pathogen **(Dadar et al. 2019).** However, isolation of the organism is time-consuming and organism management necessitates specialized laboratory equipments and highly skilled personnel to handle clinical samples and live bacteria for final recognition, speciation, and biotyping **(Kaynak et al. 2016).**

In order to overcome these challenges, polymerase chain reaction (PCR)-based tests for the quick recognition and verification of bacteria at species and biovar level, including *Brucella*, have been developed, which virtually eliminate the need for direct handling of the pathogen.

Molecular detection techniques for *Brucella* diagnosis have grown in popularity during the previous few decades. Real-time PCR, which has a minimal risk and great sensitivity, has been used to detect *Brucella.* Contamination is less likely with real-time PCR since it does not require extensive modification (**Yaran et al. 2016)**.

The presence of *Brucella* DNA in serum was verified in several studies, but this was qualified as the release of nucleic acids in serum due to the disintegration of the organisms during bacteremia. Recently, **Wareth et al. (2015)** detected *Brucella* genomic DNA by real time -PCR in the serum of infected cattle, sheep and goats in Egypt. They established that real time PCR is more sensitive and specific compared with serological tests. PCR could detect 5 fg of DNA **(Kaushik et al. 2006)**.

The aim of the present study is to (i) Detect *Brucella* infection in the serum of dairy buffaloes based on serological and molecular methods and assess the sensitivity and specificity of the commonly used tests to establish the most relevant assays for accurate diagnosis. (ii) Isolate and classify *Brucella* species bacteriologically in order to identify the most frequent field strain in dairy buffaloes.

MATERIALS and METHODS

1- Animals and samples:

Blood and milk samples were collected from buffalo dairy herds totaling 316 samples (where 290 buffaloes suffering from different reproductive disorders and a previous history of *Brucella* recovery and 26 apparently healthy buffaloes) from different rural areas belonging to the Al-Qalyubia Governorate. These investigated animals had no history of *Brucella* vaccination. Blood serum samples were examined

by different tests for the estimation of relative sensitivity and specificity. Collected milk samples were used for MRT and isolation of *Brucella* organisms.

2-Serological examination:

Clinical samples of examined buffaloes were subjected to serological assessments using MRT, RBPT and complement fixation test (CFT) according to **Alton et al. (1988)** and **OIE (2019)**. C-ELISA was performed using commercial Svanova kits according the manufacturer's protocol (Svanova Biotech AB, pat. No. 18-0004-01\06, Lot. B56923.Ex.11\12\2022, Uppsala, Sweden).

3-Isolation and identification of *Brucella* species from milk samples:

Brucella species were isolated and identified from milk samples according to **Alton et al. (1988)** and **Tantillo et al. (2003)**.

4-Molecular examination:

A. **DNA extraction:** DNA extraction from serum samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the serum sample suspension were incubated with 10 µl of proteinase K and 200 µl of lysis buffer and incubated at 56^{O}C for 10 minutes. After incubation, 200 µl of 100% ethanol were added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit. Purity and concentration of DNA was determined photometrically using a Nano Drop ND-1000 UV-Vis spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA).

B. Oligonucleotide Primers:

A.The oligonucleotide primers and Primers sequences, target gene and cycling conditions amplify IS711 gene for identification of *Brucella* to the species level were carried out according to **Bricker and Halling (1994) (**Table 1).

C-SYBR green real-time PCR amplification:

Primers were utilized in a 25- µl reaction containing 12.5 µl of the 2x QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany, GmbH), 0.5 µl of each primer of 50 pmol concentration, 5.5 µl of water, and 6 µl of DNA template. The reaction was performed in a Stratagene MX3005P real time PCR machine.

D-Analysis of the SYBR green real-time PCR results:

Amplification curves and cycle threshold (ct) values were determined by the Stratagene MX3005P software.

5-Statistical analysis:

Data analyses were carried out using a statistical software program (SPSS for Windows, Version 21.01, IBM, SPSS Inc., Chicago, USA). The agreement between different serological tests was calculated using Kappa analysis. Isolation and rtPCR were selected in this study as the "gold standard" to classify the tested animals as true positives or true negatives.

Table 1. Primers sequences, target genes and cycling conditions for SYBR green rt-PCR .

Target gene	Target agent	Primers sequences	Amplified segment (bp)	Primary denatura-tion	Amplification (40 cycles)			Dissociation curve (1 cycle)		
					Secondary denaturation	Annealing (Optics on)	Extension	Secondary denaturation	An-nealing	Final denatura-tion
1S711	*Brucella* genus	IR1 GGC-GTG-TCT-GCA-TTC-AAC-G	839	94˚C 5 min.	94˚C 15 sec.	55˚C 30 sec.	72˚C 45 sec.	94˚C 1 min.	55˚C 1 min.	94˚C 1 min.
		IR2 GGC-TTG-TCT-GCA-TTC-AAG-G								
	B. abortus	1S711-specific Primer TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT	498	94˚C 5 min.	94˚C 15 sec.	55˚C 30 sec.	72˚C 30 sec.	94˚C 1 min.	55˚C 1 min.	94˚C 1 min.
		B. abortus-specific Primer GAC-GAA-CGG-AAT-TTT-TCC-AAT-CCC								
	B. melitensis	1S711-specific Primer TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT	731	94˚C 5 min.	94˚C 15 sec.	55˚C 30 sec.	72˚C 45 sec.	94˚C 1 min.	55˚C 1 min.	94˚C 1 min.
		B. melitensis-specific Primer AAA-TCG-CGT-CCT-TGC-TGG-TCT-GA								

Reference **Bricker and Halling (1994)**

RESULTS

Table 2. Results of MRT and bacteriological culture of milk samples from examined buffaloes.

Examined animals	MRT		Bacterial isolation	
	No.	%	No.	%
Buffaloes suffering from reproductive disorders (N=290)	59	20.34	47	16.21
Apparently healthy buffaloes (N=26)	3	11.54	-	-
Total (N=316)	62	19.62	47	14.87

Results showed that all of the 47(14.87%) isolated strains were identified and biotyped as *Brucella melitensis* biovar 3. A total of 62 (19.62%) positive reactors were found using the MRT.

Table 3. Serological profile of brucellosis based on different serological tests and rtPCR on serum samples among examined buffaloes.

Examined animals	RBT		cELISA		CFT		rt PCR	
	No	%	No	%	No	%	No	%
Buffaloes suffering from reproductive disorders (N=290)	53	18.28	53	18.28	53	18.28	54	18.62
Apparently healthy buffaloes (N=26)	2	7.69	1	3.85	-	-	-	-
Total (N=316)	55	17.41	54	17.09	53	16.77	54	17.09

The obtained results showed that RBT, cELISA, CFT, and rtPCR, seropositivity were founded in 55 (17.41%), 54 (17.09%), 53 (16.77%) and 54 (17.09%) of clinical samples, respectively.

Table 4. Diagnostic parameters of different tests in comparison with bacterial isolation as a gold standard.

Tests	Bacterial isolation		Diagnostic parameters				
	Positive 47	Negative 269	Se	Sp	PPV	NPV	Agreement, k value (± SE)
MRT Pos 62	44	18					0.768
Neg 254	3	251	93.6	93.3	70.97	98.8	SE 0.048
RBT Pos 55	44	11					0.837
Neg 261	3	258	93.6	95.9	80	98.9	SE0.042
cELISA Pos 54	43	11					0.823
Neg 262	4	258	91.5	95.9	79.6	98.5	SE 0.047
CFT Pos 53	43	10					0.834
Neg 263	4	259	91.5	96.3	81.1	98.5	SE 0.043
rtPCR Pos 54	45	9					0.881
Neg 262	2	260	95.7	96.7	83.3	99.2	SE 0.038

Kappa < 0: No agreement, Kappa between 0.00 and 0.20: Slight agreement, Kappa between 0.21 and 0.40: Fair agreement, Kappa between 0.41 and 0.60: Moderate agreement, Kappa between 0.61 and 0.80: Substantial agreement, Kappa between 0.81 and 1.00: Almost perfect agreement.

Obtained results showed the sensitivity, specificity, positive predictive value, and negative predictive value of MRT, RBT, cELISA, CFT and rtPCR in comparison with bacterial isolation as a gold standard were calculated as 93.6 %, 93.3%, 70.97 %, and 98.8 %, respectively for MRT with a substantial aggreement of 0.768, with regard to RBT were 93.6 %, 95.9%, 80 %, and 98.9 %, respectively, with an almost perfect agreement of 0.837, with reference to cELISA were estimated to be 91.5 %, 95.9%, 79.6% and 98.5 %, respectively, with an agreement of 0.823, while for CFT were 91.5%, 96.3%, 81.1%, and 98.5%, respectively, with an agreement of 0.834, for rtPCR were 95.7%, 96.7%, 83.3% and 99.2%, respectively, with almost perfect agreement of 0.881.

Table 5. Diagnostic parameters of different tests in comparison with real-time PCR as a gold standard.

Tests	rtPCR		Diagnostic parameters				
	Positive 54	Negative 262	Se	Sp	PPV	NPV	Agreement, k value(± SE)
MRT Pos 62	53	9					0.895
Neg 254	1	253	98.2	96.6	85. 5	99.6	SE 0.033
RBT Pos 55	52	3					0.945
Neg 261	2	259	96.3	98.9	94.6	99.2	SE 0.025
cELISA Pos 54	51	3					0.933
Neg 262	3	259	94.4	98.9	94.4	98.9	SE 0.027
CFT Pos 53	52	1					0.966
Neg 263	2	261	96.3	99.6	98.1	99.2	SE 0.019

Kappa < 0: No agreement, Kappa between 0.00 and 0.20: Slight agreement, Kappa between 0.21 and 0.40: Fair agreement, Kappa between 0.41 and 0.60: Moderate agreement, Kappa between 0.61 and 0.80: Substantial agreement, Kappa between 0.81 and 1.00: Almost perfect agreement

Results showed the sensitivity, specificity, positive predictive value, and negative predictive value of MRT, RBT, cELISA and CFT in comparison with rtPCR as a gold standard were calculated to be 98.2%, 96.6%, 85.5% and 99.6%, respectively for MRT, for RBT were 96.3 %, 98.9%, 94.6 %, and 99.2 %, respectively, with an almost perfect agreement of 0.945, while for cELISA were 94.4%, 98.9%, 94.4%, and 98.9%, respectively, and for CFT were 96.3%, 99.6%, 98.1% and 99.2%, respectively. with almost perfect agreement 0.966.

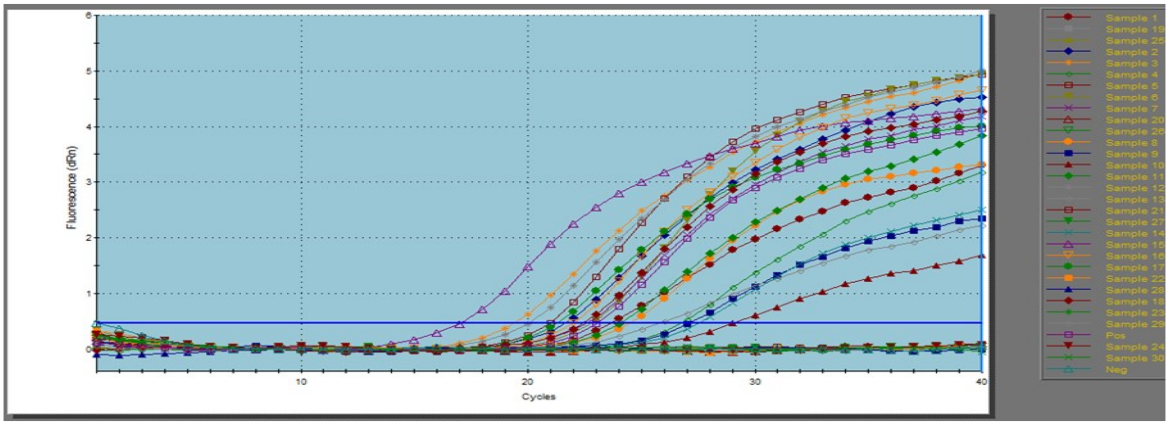

Figure 1. Real-time PCR amplification curves of the *Brucella* genus, showing - Positive samples and Positive control (upper curve); - Negative samples and Negative control (lower curve).

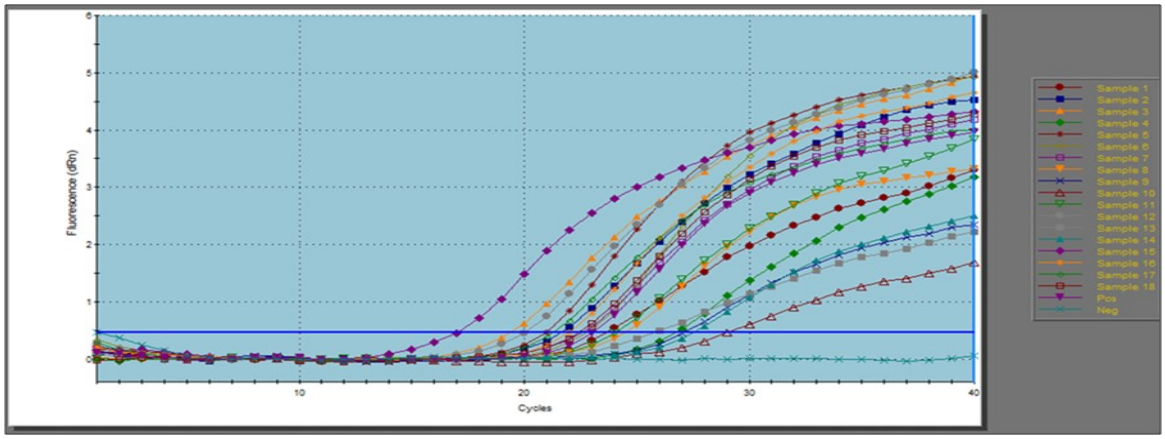

Figure 2. Real-time PCR amplification curves of *B. melitensis* specific primer, showing – positive samples and Positive control (upper curve); - Negative control (lower curve).

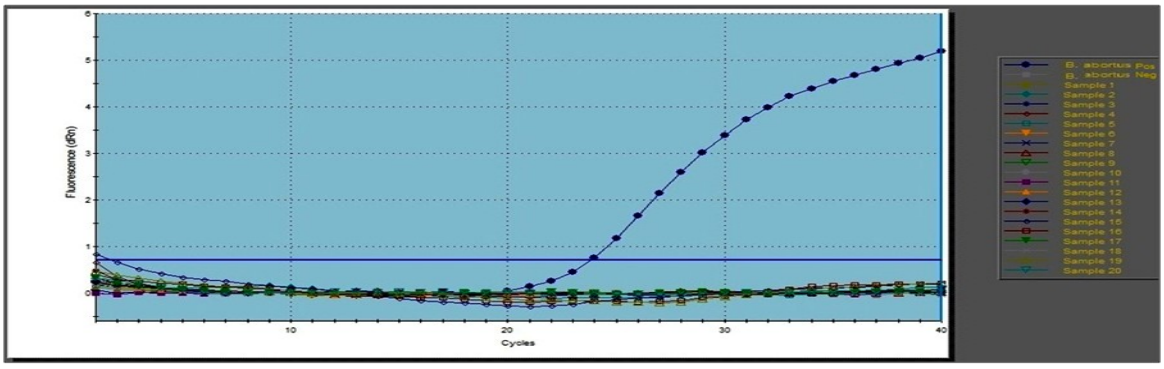

Figure 3. Real-time PCR species specific for *B. abortus* specific primer, amplification curves showing – negative Samples and Negative control (lower curve).

DISCUSSION

In this study, bacteriological examination of buffalo milk samples revealed the isolation of *Brucella* microorganisms from forty-seven buffaloes (14.87%) out of 316 milk samples examined. Decreased sensitivity of the culture technique may be related to the fastidious nature of *Brucella* **(Alton et al. 1988).** Failure of isolation can occur when the clinical sample contains a limited number of viable bacteria or when the clinical sample is heavily contaminated **(Seleem et al. 2010).** Intermittent *Brucella* shedding in milk is another major limiting factor **(Wernery et al. 2007).** All of the 47 isolated strains were identified and biotyped as *Brucella melitensis* biovar 3 (Table 2). This could be due to mixed populations of bovines, and small ruminants, which has raised the hazard of brucellosis since sheep and goats act as primary hosts for *B. melitensis*, while bovines act as spilling over hosts **(El-Wahab et al. 2019** and **Aliyev et al. 2022).** According to the obtained bacteriological findings, it can be concluded that *B. melitensis* biovar 3 remains the predominant strain identified among buffaloes under local conditions in Egypt that comes into agreement with previous studies **(Samaha et al. 2008, Menshawy et al. 2014, Hosein et al. 2017, Hosein et al. 2018** and **Soliman et al. 2019)**.

Diagnostic parameters of different tests were calculated in this study using *Brucella* isolation as the gold standard. The employ of serological tests is a technique planned for indirect diagnosis of the disease; however, at present the sensitivity and specificity of serologic tests are not at the desired level because of false positive and/or negative reactions (**Cutler, 2006).**

In this investigation MRT, RBT, cELISA, CFT, and rtPCR, seropositivity were founded in 62 (19.62%), 55 (17.41%), 54 (17.09%), 53 (16.77%) and 54 (17.09%) of clinical samples, respectively (Tables 2&3). As the findings of these tests showed marked variation that resulted in misclassification, it was important to conclude that more than one diagnostic procedure is required for the diagnosis of brucellosis -positive animals, especially for epidemiological purposes.

A total of 62 (19.62%) positive reactors were found using the Milk Ring Test (Table 2). MRT has a sensitivity of 93.6 %, which is close to that of RBT, but the specificity is the lowest of all the tests in this study 93.3 %. With MRT there is the lowest positive predictive value of 70.97 % with a substantial agreement of 0.768 and 98.8% negative predictive value (Table 4). These findings are consistent with those of **Trangadia et al. (2010)** and **Almashhadany (2021),** who stated that the MRT may be used to scan a herd of animals and that such a reaction has adequate sensitivity and specificity to identify *Brucella* on individual basis.

The highest positive reactors were reported with RBT 55(17.41 %), as shown in (Table 3). The sensitivity, specificity, positive predictive value, and negative predictive value of RBT were calculated as 93.6 %, 95.9%, 80 %, and 98.9 %, respectively, with an almost perfect agreement of 0.837 (Table 4). The test is similar to MRT in terms of sensitivity, but it is more specific. **Hosein et al. (2017)** found that buffered *Brucella* antigen tests have higher sensitivity, but less reliable specificity, resulting in fewer false negatives and a significant number of false positives. Finally, the World Organization for Animal Health (OIE) prescribed using the RBT as an approved screening test and the complement fixation test as a confirming test for brucellosis control at the national or local level **(OIE 2016).**

According to Table (3), cELISA detected 54 (17.09%) reactors. The sensitivity, specificity, positive predictive value, and negative predictive value of cELISA were calculated as 91.5 %, 95.9%, 79.6% and 98.5 %, respectively, with an agreement of 0.823 (Table 4). The Monoclonal antibodies (MAb) targeting one of the epitopes of *Brucella* spp. has been demonstrated to have similar specificity, but poorer sensitivity than the RBPT; this is steady with **Munoz et al. (2005).** Consequently, the **OIE (2018)** recommended that positive c-ELISA reactions should be subjected to an appropriate confirmatory test. The test also lowers, but not completely eliminates, false positive serologi-

cal reactions caused by cross-reacting bacteria and reduces, but not completely eliminates, reactions caused by vaccination.

Table (4) shows the higher specificity of CFT (96.3%) than RBPT, indicating that the RBPT positive samples ought to be confirmed by this test. With an agreement of 0.834, the CFT's sensitivity, specificity, positive predictive value, and negative predictive value were estimated to be 91.5%, 96.3%, 81.1%, and 98.5%, respectively, (Table 4). These results showed that CFT shares the same sensitivity as c-ELISA but is more specific than it. **Al Dahouk et al. (2003)** considered that CFT should be utilized just as a confirmatory test and it was noticed that, in functional terms, sensitivity, and specificity could vary broadly. This test is considered a high-quality test when correctly used, but it has lots of practical drawbacks, such as being time-consuming and difficult to standardize **(Abernethy et al. 2012).** The screening test results should be confirmed using complementary tests, which are measured more dependable for confirming and verifying suspicious results so acquired **(Nammalwar et al. 2009).**

Regarding molecular detection approaches to detect at the genus level, real-time PCR identified 54 positive reactors (17.09%) (Table 4), all of which were identified as *B. melitensis* (Figures 1 & 2).

Table (4) shows the highest sensitivity (95.7%) and specificity (96.7%) of rtPCR. The positive predictive value and negative predictive value of rtPCR were estimated at 83.3% and 99.2%, respectively, with almost perfect agreement of 0.881. In this study, rtPCR showed better positive predictive value than the rest of the immunoassays. This minimizes the possibility of giving false positive animals in comparison with any other of the evaluated immunoassays. This result agrees with **Hassanian and Wahid (2012)** and **Wareth et al. (2015),** who found that PCR should be regarded as the gold standard diagnostic method for detection of Brucella infection as this method had a higher sensitivity and specificity for detection of brucella infection when compared to other serological methods.

Saleha et al. (2014) reported that PCR is needed for an accurate diagnosis of Brucellosis in livestock diagnostic laboratories. Furthermore, as PCR is a more reliable test when its sensitivity is considered for antigen detection in clinical samples than antibody detection, it may be included as a routine screening test in clinical practice in farm animals irrespective of its high cost as compared to conventional tests in order to reduce financial losses.

Since some animals with negative cultures may be truly infected, the sensitivity and specificity of a serological test cannot normally be determined on the basis of bacteriological data. The positive predictive value, negative predictive value, and relative sensitivity, and specificity of serological assays under concern were calculated in this study based on rtPCR as the gold standard (Table 5).

MRT had the highest sensitivity (98.2 %), but the lowest specificity (96.6 %) of all the tests in this investigation (Table 5). MRT appears to be technically sound as a dairy herd screening test, according to **Beleta and Khafagi (2021).**

The relative sensitivity, relative specificity, positive predictive value, and negative predictive value of RBT were calculated to be 96.3 %, 98.9%, 94.6 %, and 99.2 %, respectively, with an almost perfect agreement of 0.945 (Table 5). These findings support those of **Aggad (2003),** who concluded that RBPT is more effective and sensitive tool in screening and detecting *Brucella* infection. This implies that RBPT is still the most accurate serological test for surveillance and eradication on a wide scale **(Garin-Bastuji et al. 2006).**

Competitive ELISA verified the lower relative sensitivity (94.4%) when compared to RBT (Table 5), although, the relative specificity of the cELISA was (98.9%). **Mustafa et al. (2012)** reported that cELISA is a highly specific and sensitive diagnostic assay as it directly detects antibody and has minimum or no false positive reactions of agglutination test and its results afford an epidemiological tool for investigating the infective status.

The CFT had the greatest results in terms of test validation criteria, such as; relative sensitivity, relative specificity, PPV, and NPV (96.3%), (99.6%), (98.1%) and (99.2%), respectively, with almost perfect agreement 0.966. The CFT is a generally utilized test, and has been viewed as the most particular serological test for detection of brucellosis; therefore it is a prescribed test for global trade **(OIE, 2018).**

CONCLUSION

MRT and RBT are still suggested for screening brucellosis in dairy buffaloes, and CFT is recommended for confirming infection in individual animals. Using rtPCR on serum as an additional diagnostic tool for detecting and diagnosing *Brucella* infection should be evaluated in addition to other serological tests that can progress and overcome the limitations of different immunoassays. According to bacteriological testing, *B. melitensis* biovar 3 is still the prevalent field strain found in the milk of buffaloes suffering from reproductive problems.

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