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Novel *Brucellosis* Diagnostic Tool by Using Microtiter and Reading the Results by Naked Eye in Less Than One Hour

Talaat Al. Mohammed
Medical Laboratory, Faquos Fever Hospital, Ministry of Health. Egypt
*E. mail: drtlat44@gmail.com

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ABSTRACT

Background: *Brucellosis* is continuing to be a major public health concern globally; diagnosis of brucellosis should be consistent with the laboratory test and clinical appearance. Therefore; anew serological test must be easy in applied and the results can be obtained within a short time. **Aim of study**: This study amid to establish a highly accurate universal diagnostic test detect *brucellosis* by microtiter in less than one hour, read the result by naked eye. **Material and methods:** The study was conducted in Fakous and Zagazig Fever Hospital and some hospitals in (Egypt), 2020 to 2023. Out of (1292) patients diagnosed for clinical *brucellosis*. 323 healthy case of different age and sex were also studied as control. All participants were clinical examination, and lab investigations. **Result**: Among (1292) clinically suspected cases (54%) females, (46%) males. Clinically have a fever case with *brucellosis* and age between 15 to 75 years. **Conclusions**: The developed microtiter assay is highly sensitive and specific, with minimal time of reaction. Moreover; this assay is rapid, easy to apply, cost effective, and requires no laboratory set ups for its application and can be deployed for on field testing of differential diseased cases. was concluded that the solution to the problems of epidemiological investigation of *Brucella* outbreaks to ensure precise diagnosis will require employing a microtiter test possessing different tasks of the immune response. Predictive values were found to be appropriate high sensitivity, specificity during screening. Therefore; anew diagnosis by microtiter is very reliable and accurate diagnosis of *brucellosis*.

INTRODUCTION

Brucellosis is a zoonotic disease and continues to be a major public health concern globally, and authors further concluded that when using the ELISA test, we must note: sensitivity of ELISA is generally high and the specificity is lower. ELISA test is usually performed less well and should be get the good measure of taking in consideration the epidemiological background when working in regions of endemicity Franco *et al.*, (2007). Almost all laboratories put in an application serological test that do not give suitable sensitivity and specificity for this organism. ELISA methods are sensitive and high specificity Vakili *et al.*, (2010).

Authors further concluded that sensitivity of ELISA is increased when the levels of IgG and IgM against *Brucella* are considered in combination and that serology results should be interpreted in tandem with clinical history, symptoms of patients and other diagnostic tests. Therefore; we believe that our results are in the clinical interest of the physicians who find it challenging to interpret different patterns of serology results by ELISA Fadeel *et al.*, (2011). In ELISA technique, is a very specific and sensitive diagnostic test since it directly discovered antibody and has very little or no false positive reactions of agglutination test, but is high expensive in poor counters Jabbar *et al.*, (2012). It has been reported Geresu and Kassa (2016), that ELISA test is most used as a test for the diagnosis of *brucellosis*. The test measures antibodies give a better analysis during investigation of *Brucellosis*, but not used in the poor counters. In many countries, micro titer was found to be more advanced to some test like slide and tube agglutination in efficacy. Altogether, it was shown a suitable substitute for diagnosis of human *brucellosis* Jimmenez de Bagues *et al.,* (1992). Since the microtiter has the priority of being able to method a great number of samples in the short time Park *et al*., (2005). However, analysis of *brucellosis* by microtiter, it was given that, microtiter was more sensitive, simpler to perform, and easier than any test Kimura *et al.,* (2008). In a study by Sareyyupoglu *et al.,* (2010), who reported that, *brucella* antibodies were investigated in blood by microtiter, and microtiter was determined as a fast, reliable, and economic test. On evaluation of *brucellosis* by microtiter. The heightened tube agglutination and microtiter are the most used for the detection of *Brucellosis*. Tube agglutination has technical disadvantages that limit its wide spread use in the field evaluation of the *Brucellosis*; additionally, it is time-consuming and slow in terms of the performance and measurement, and zone phenomena might occur. Oncel, *et al.*, (2005). On evaluation of brucellosis by MAT, it was shown that MAT was more sensitive, simple and rapid diagnosis of brucellosis. MAT has a greater accuracy than that of the TAT, and ELISA as diagnostic tools for brucellosis Welch *et al.*, (2010). A study by R. Flores-Castro and Carmichael (1978), has shown that the tube agglutination test and slide agglutination test many times give false results because of cross-reactions with other disease. One of the problems that is always recurring, in tubes in which agglutination has occurred before dilution because of the optimal antibody antigen ratio, agglutination may not be detected because of the relative increase of antibodies against antigens. Therefore; the test becomes positive while it is negative in the first tubes Chu and Weyant (2003). The study revealed that, slide and tube agglutination can give false positive reactions with sera from patients infected with any disease without developing disease, and give low sensitivity and give high number of false negatives in cases of chronic disease WHO (2006). In several patients the zone phenomenon effects are rarely reported in microtiter and the results obtained during titration are correct In a study by Knudtson and Fetters (1990). who reported that, the zone phenomenon generally refers to a false-result response appear from cases in which high antibodies titer reacts with the antigen antibody network formation. The zone phenomenon occurs when undiluted sample French *et al.*, (2009). Diagnosis of brucellosis is difficult due to the restriction of diagnostic test and the direction of *Brucella* spp to produce nonspecific, false clinical signs and making prevention essential. In *brucellosis* control and eradication Programs, by WHO it was shown that guidelines recommended the use of the sensitive rapid screening test, SAT, TAT, ELISA, but the card agglutination test that detects both agglutinating and non-agglutinating

antibodies, give positive/negative results. Moreover; commercialization of the serological kit for SAT, validated for human diagnostics, has recently been discontinued, making it necessary to identify new serological diagnostic solutions Corbel (2024). In this study, aimed to establish an accurate and sensitive MAT technique in a shortly time for the diagnosis of *brucellosis*, when compared with ELISA, SAT and TAT. MAT uses smaller quantities of antisera, antigen and buffer. Therefore; MAT is more sensitive, economical and the end results is read by naked eye in less than one hour.

MATERIALS AND METHODS

Patients:

The study was conducted at the Fakous and Zagazig fever hospital and some hospitals in (Egypt), from July 2020 to September 2023. (1292) human sera from suspected cases of *brucellosis*. Diagnosis for these cases was based on clinical and serological evidence together with professional.323 healthy individuals of different age and sex were also studied as control. All reagents are ready to use. The results of the tests is obtained in less than one hour. Standard microtiter method: The kit is based on MAT using antigen of *Brucella*. All reagents were thawed to 25°C and mixed by gentle vertexing before use .

The Material Provided in The Microtiter Test :

Brucella antigens suspensions had commercially available in 5ml. Working reagents contain dissolved (3.2 g NaCl), (sodium citrate: 7.9 g), (280 μl HCl), 100 ml. D.W. Sterile test tube's micropipettes, tips and microtiter plate wells.

Procedure:

Step1: Bring all reagents and serum samples at room temperature before testing. Shake well and mix antigen well before dispensing. Separate microtiter plate to two part, In part of *brucella* abortus add (100 μl) of buffer solution in well number 1 (Case1 C), add (50 μl) of buffer solution in well 2 to well 6 and add (15 μl) of serum sample in well number (1) mix in well number (1) transfer (50 μl) from the well number(1) into well (2) to (6) and discard (50 μl) out .

Step 2: Shake the *brucella* abortus antigen suspension well before use and add (15μl) of this suspension to each well (1 to 6).

Step 3: Mix the material of the plate very carefully by stirring laterally on the side of the microtiter plate. Keep the plate stationary at room temperature (20-25 °C) in a flat position away from sources of vibration, stirring. Read the result after less than one hours by naked eye, providing that the plate remains motionless, protected from vibration.Make this step in part 2 of microtiter plate well with *brucella meltensis.*

Result Interpretation:

Positive Reaction: Ring formation at the bottom of the wells.

Negative Reaction: No ring

Validation Protocol:

Sensitivity : It is the capacity of the test to detect diseased patients, when compared with the gold standard test Sensitivity = TP / (TP + FN) x 100 by Trevethan (2017).

Specificity: It is the capacity of the test to detect non-diseased patients, when compared with the gold standard test Specificity= TN / (TN+ FP) x 100.

Sensitivity, specificity, Positive predictive value and Negative predictive value were determined according to the method described by Trevethan (2017).

Stability : Stability of a measured in a specimen is a function of the property variation over time in specific storage conditions, and is usually simplified to stability limits Gómez-Rioja *et al.,* (2018).

Accuracy : By a serodiagnosis test high specificity and sensitivity. to produce high performing designer immunological reagents. Consequently, serodiagnosis can be conducted more accurately at a lower cost (Mayara *et al.,* 2020).

Standard ELISA method:

Procedure : Distribute 90 μl of diluted wash solution (1:10) in each well of the plate. Mixed the contents within each well

by gently shaking the plate. Covered the plate with a lid and incubated for 60 min at 37°C in a humid chamber. Washed each well with approximately 300 *µl* wash solution 3 times. Aspirated liquid contents of all the wells after each wash. divided100 *µl* conjugate into each well. Covered and incubated the plate for 60 min at 37°C in a humid chamber. Washed each well and aspirated the liquid contents of all the wells. distributed 100 *µl* of into each well and incubated the substrate at 18-26°C for 15 min. Stopped the color reaction by adding 100 *µl* of stop solution per well. Read the results at a wavelength of 450 nm.

Standard Tube Method:

Procedure : Put 10 test tubes were placed in a rack then 0.9 ml saline was delivered in the first test tube and 0.5m1 in each of the remaining test tubes. Add 0.1m1 of serum to the first test tube. After mixing, 0.5m1 of the diluted serum was transferred to the second test tube, and so on until the contents of tube 10 were mixed. Add an antigen another tube was added to the series containing 0.5m1 saline. Then 0.5 m1 B. antigen. shaking the rack well, it was placed in a 37 °C water bath for 24 hours and read results.

Standard Slide Method:

Procedure : Using an applicator stick, the serum and antigen in each square were mixed. The slide was rocked gently no longer than 3 minutes. After that agglutination was detected.

Statistical Analysis:

Microsoft Excel program and data were analyzed using the Statistical Package for Social Science (SPSS) version 2019 software program (Boyd *et al.*, 2019).

RESULTS

Four serological test methods revealed that patients had the highest *brucellosis* prevalence among the (1292) sera samples tested. Among the samples tested for. ELISA, MAT, TAT and SAT. Enzyme Linked Immunosorbent Assay *brucella* abortus (ELISA BA IgM) and Enzyme Linked Immunosorbent Assay *brucella meltensis* (ELISA BM IgM) 53.2% and 48.9% respectively. MAT BA and MAT BM were 54.2% and 50.5% respectively. TAT BA and TAT BM were 39.3% and 37.2% Finally SAT BA and SAT BM were 36.8% and 39.6%. respectively (Fig. 1).

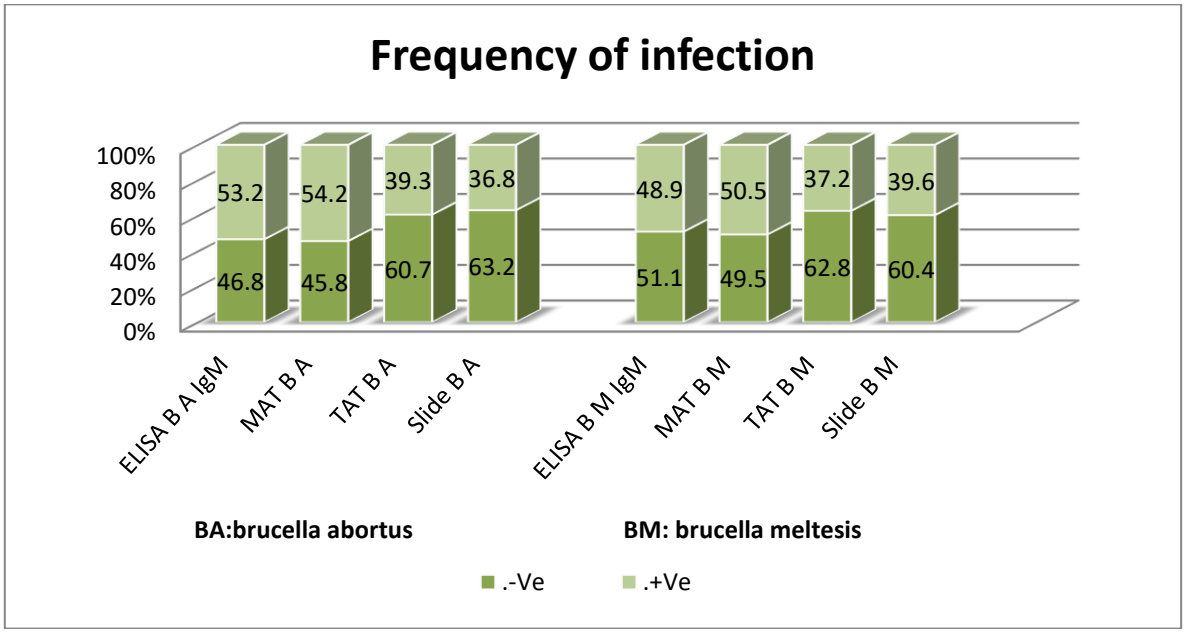

Fig. 1: The rate at which occurs frequency of infection between of the studied cases by used different methods ELISA, MAT, SAT and TAT.

In this study, the level of positivity in the MAT results ranged from 1/160 to 1/2560. A similarity was found in positivity between the MAT and ELISA results. However, according to the results generated using serially diluted samples, MAT appeared to be more successful in detecting weak positives (Table 1).

Out of (1292) patients studied, shows that, MAT BA had a sensitivity of 100%, specificity 98.1%, PPV 98.3%, NPV 100% and accuracy of 99.1 %. while TAT BA had sensitivity 42.4%, specificity 64.2% PPV 57.5%, NPV 49.5% and accuracy 52.6%. Finally, SAT BA had a sensitivity of 38.4%, specificity 64.9%, PPV 55.5%, NPV 48.0% accuracy 50.8% of the patients (Table 1).

In the same table, shows that, MAT BM had a sensitivity of 98.7%, specificity 95.8%, PPV 95.7%, NPV 98.8% and accuracy of 97.2 %. while TAT BM had sensitivity 40.5%, specificity 66.1% PPV 53.3%, NPV 53.7% and accuracy 53.6%. Finally, SAT BM had a sensitivity of 39.9%, specificity 60.6%, PPV 49.2%, NPV 51.3% accuracy 50.5% of the patients.

Table 1: Determination of Sensitivity, Specificity, PPV, NPV and Accuracy in diagnosis of *brucellosis* by different methods ELISA IgM, MAT, TAT and SAT.

Laboratory test		Sensitivity	Specificity	PPV	NPV	Accuracy	P
ELISA B A IgM	MAT B A	100%	98.01%	98.3%	100%	99.1%	<0.001**
	TAT B A	42.4%	64.2%	57.5%	49.5%	52.6%	0.22 NS
	Slide B A	38.4%	64.9%	55.5%	48.0%	50.8%	0.543 NS
ELISA B M IgM	MAT B M	98.7%	95.8%	95.7%	98.8%	97.2%	<0.001**
	TAT B M	40.5%	66.1%	53.3%	53.7%	53.6%	0.222 NS
	Slide B M	39.9%	60.6%	49.2%	51.3%	50.5%	0.930 NS

Table 2: Classification of different methods MAT, TAT and SAT according to low and high titers.

Antibody titers	MAT No of cases (1292)				TAT No of cases (1292)				SAT No of cases (1292)			
	MAT BA		MAT BM		TAT BA		TAT BM		SAT BA		SAT BM	
	positive (+Ve)	negative (-Ve)	positive (+Ve)	negative (-Ve)	positive (+Ve)	negative (-Ve)	positive (+Ve)	negative (-Ve)	positive (+Ve)	negative (-Ve)	positive (+Ve)	negative (-Ve)
1/80	0	592	0	640	0	784	0	812	0	816	0	780
1/160	348	0	324	0	384	0	360	0	392	0	456	0
1/320	264	0	248	0	92	0	100	0	80	0	44	0
1/640	36	0	48	0	32	0	20	0	4	0	12	0
1/1280	28	0	16	0	0	0	0	0	0	0	0	0
1/2560	24	0	16	0	0	0	0	0	0	0	0	0

In all 1292 cases the, MAT BA test was positive in titer 1/160 (348 case), 1/320 (246 case), 1/640 (36 case), 1/1280 (28 case), 1/2560 (24 case). In which the MAT B M were 1/160 (324 case), 1/320 (248 case), 1/640 (48 case), 1/1280 (16 case), 1/2560 (16 case), as presented in (Table 2 & Fig. 2).

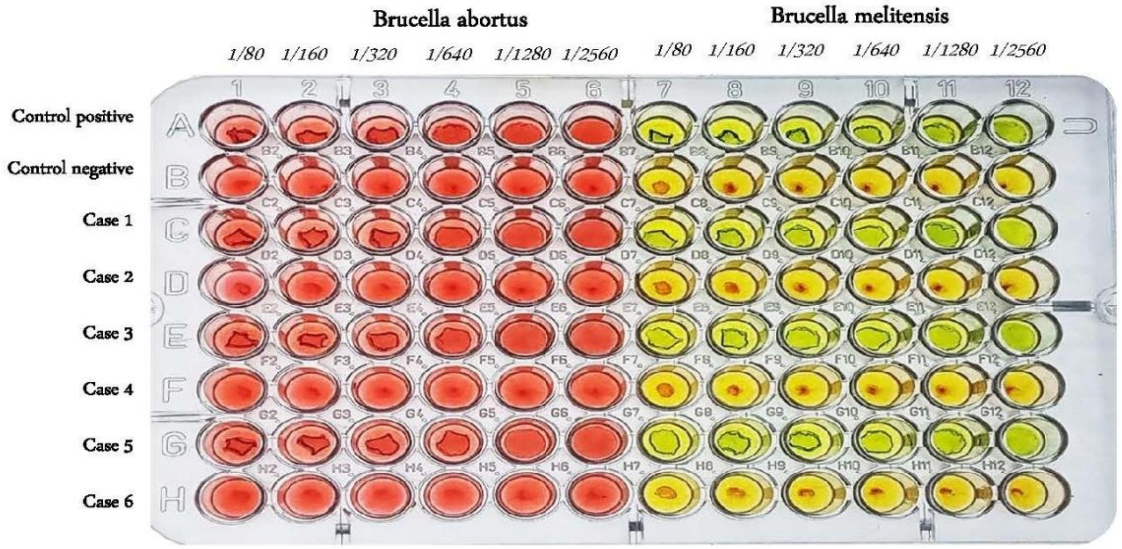

Fig.2: determination of *brucella* abortus and *brucella* meltensis (after the reaction) : A control positive, B. control negative, **case** 1. *brucella* abortus 1/1280 and *brucella* meltensis 1/1280, **case** 2. negative, **case** 3. *brucella* abortus 1/640 and *brucella* meltensis 1/2560, **case** 4. negative, **case** 5. *brucella* abortus 1/2560 and *brucella* meltensis 1/2560, **case** 6. negative.

DISCUSSION

In this study, easy, simple, low resource tool development was our goal, giving patients hope for a better life. Our study has the possibility to change the way of diagnosis of *brucellosis* is performed, providing instead a portable, reliable and cost-effective solution for test, unlike current diagnostic methods, which require more, this test offers a quicker solution. Which is crucial for rapidly determining treatment plans, capabilities traditional diagnostic approaches cannot achieve. Not only can our blood test provide same day results, this test can be quickly performed in any lab and does not require specialized training or equipment needed. With the number of *brucella* infection cases rising each year, there's a pressing need for quick and precise diagnostic methods and providing results in as little as one hours. But we envision this as a tool that could be deployed worldwide, as the future of early detection of *brucellosis*. in all the world there is a high demand for rapid screening test that can decrease the turnaround time, cost, and limits of quantitation of existing. Our method targets not only diagnosis of *brucellosis* but diagnosis other types of infection diseases.

This study is in agreement with Damp *et al.*, (1973), who found that, MAT and ELISA are the most widely used laboratory test for the detection of *Brucellosis* antibodies in patients. However; MAT, is suitable because a larger number of samples can be processed together. Also; Allan *et al.*, (1976) found that; MAT is accurate most sensitive diagnosis for *brucella* infection as compared with TAT and SAT agglutination test. In our study, an using ELISA the gold standard, the sensitivity of MAT BA in patients was 100% ppv 89.6 % npv 100% respectively. Specificity for MAT BA in patients was 93.8% respectively. the sensitivity of MAT B M in patients was 93.4% ppv 86.8 % npv 97.0% respectively. Specificity for MAT BM in patients was 82.8% respectively. In accordance with this result, findings of each of Smits *et al.*, (2001) and Dey *et al.*, (2006) found that; the high sensitivity and specificity was detected in MAT. The high accuracy of microtiter may be due to its ability to differentiated amounts of antibodies in the start of infection, which SAT and TAT does not. results are gained

almost at once and visual check with the naked eye. As evident from our present data, Lucero *et al.,* (2005). Reported that; *Brucellosis* symptomatic infections in humans may be more diffuse than has been estimate. Epidemiological studies may help increase the comprehension of the prevalence of *Brucellosis*, and they can understand preventive measures for decrease human exposure to the bacteria, by applied a new diagnostic method. A study by Gómez *et al.,* (2008). Has shown that; there are also index that ELISA test do not have suitable specificity to be used as diagnostic tools, and must be compared these results with those obtained compared with the different diagnostic method. However; diagnosis of *Brucellosis* by ELISA generally has determining antibodies specific to *Brucella* compared to the other serological test. And Keid *et al.,* (2008). Found that, importance of using MAT test for diagnosis of *brucellosis,* and it was concluded that MAT detected more samples as positive among these tests TAT and SAT, and to develop a serological diagnosis method that is faster and easier to perform. Diagnosis of *Brucellosis* is generally to search for discovery of infected cases preserve false positive cases to the smallest amount of level. Therefore, the idea is to use series of tests including tests with good sensitivity, specificity NPV and PPV to ensure the presence of the disease Chachra *et al.,* (2009). A study by, Varshochi *et al.,* (2011). Reported that, old serological test, tube and slide agglutination, have been shown to produce nonspecific reactions with *brucellosis*. To avert such nonspecific reactions, MAT test has been used for *brucellosis* detection. Therefore; the present study was conducted to correct the *brucellosis* diagnostic and special power of the MAT test. And Getachew *et al.,* (2016). found that; the MAT had a better specificity and sensitivity for discovery when compared to other tests.

Although, various serological tests are available, slide and tube agglutination no appropriate in all studies due to difference of their sensitivity and specificity Matope *et al.,* (2011); Mert *et al.,* (2003); OIE, (2018). However; high sensitivity of ELISA and MAT in diagnosis rates in some patients with low diffusion, and determine the diagnosis of *brucella* infection, when compared the results with SAT and TAT and estimate the different diagnostic method of *brucellosis* Pereira *et al.,* (2020). While these test TAT and SAT have been instrumental in identifying *Brucella* species and confirming infection, they often suffer from limitations such as low sensitivity, cross reactivity with other pathogens and high negative predictive value (NPV). However; their results require interpretation that is often difficult and frequently inconclusive Yagupsky *et al.,* (2020). However; these tests SAT, TAT suffer with uncertainties in the accuracy false positive and false negative results may occur with these tests, due to the difficulties of carrying out validation studies Mol *et al.,* (2020), representing a major challenge in laboratory investigations of *brucellosis* Santos *et al.,* (2021).These join present and cost effective tests presents an interesting anew diagnosis . To address this, optimize tests performance while maintaining sensitivity and reducing false positive particularly in resource limited countries Xu *et al.,* (2023). Increase antibodies in a sample can stop the inter reaction between antigen-antibody and next agglutination reaction, leading to a false result, which is known as a prozone Tizard (2004). Therefor; Endpoint titer agree with the highest serum dilution, as samples by compared with the negative controls Triola (2005). Microtiter play an important role in treatment of increase antibodies and reduce the zoophenomenn effect, sample can be diluted more. False results agree to an increase in the concentration of antibodies against a specific antigen. when antibodies concentration much higher than the antigen the density may inhibit agglutination Buzgan *et al.,* (2007). So, we counsel the routine use of MAT in the test of the serum of all patients with symptoms of *brucellosis*.

It is also important to note that there is neither a combined method for serological diagnosis of B. nor a means to standardize the antigens in any tests. So the MAT is the best in diagnosis.

Conclusions:

The developed microtiter assay is highly sensitive and specific, with minimal time of reaction. Moreover; this assay is rapid, easy to apply, cost effective, and requires no laboratory set ups for its application and can be deployed for on field testing of differential diseased cases. was concluded that the solution to the problems of epidemiological investigation of *Brucella* outbreaks to ensure precise diagnosis will require employing a microtiter test possessing different tasks of the immune response. Predictive values were found to be appropriate high sensitivity, specificity during screening. Therefore; anew diagnosis by microtitre is very reliable and accurate diagnosis of *brucellosis*.

Abbreviations:

MAT: Micro titer agglutination, **MAT BA**: Micro titer agglutination brucella abortus, **MAT BM**: Micro titer agglutination brucella meltensis, **ELISA:** Enzyme Linked Immunosorbent Assay, **ELISA BA IgM:** Enzyme Linked Immunosorbent Assay brucella abortus , **ELISA BM IgM:** Enzyme Linked Immunosorbent Assay brucella meltensis , **TAT:** Tube agglutination test, **TAT BA:** Tube agglutination test brucella abortus **TAT BM:** Tube agglutination test brucella meltensis, **SAT:** Slide agglutination test, **SAT BA:** Slide agglutination test brucella abortus, **SAT BM:** Slide agglutination test brucella meltensis, **SPSS:** Statistical Package for Social Sciences, **NPV**: negative predictive value, **PPV**: positive predictive value, **WHO:** World health organization

Declarations:

Ethical Approval: This research was approved by ethics committee of Faculty of Medicine, Zagazig University (ZU-IRB#11334-26-12-2023).

Conflicts of Interest: The author declares no conflicts of interest.

Authors Contributions: The author contributed towards the study design, experiment execution, data analysis, and manuscript drafting.

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Availability of Data and Materials: All datasets analyzed and described during the present study are available from the corresponding author upon reasonable request.

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