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Original article

Molecular Diagnosis and Antibodies Tests for Brucellosis Detected in Aborted and Apparently Healthy Sheep and Goats in and around Al Bayda City, Libya

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ABSTRACT

The goal of the current research was to evaluate antigen and antibody reaction's method in comparison with real time PCR for detection of brucella agent in sheep and goat. Therefore, seven hundreds and twenty-eight blood and milk samples were isolated from sheep and goats apparently healthy and suspected brucellosis brought to the Veterinary Clinical Complex from areas in and around Al Bayda, Libya. Blood samples were subjected to serological tests including Rose Bengal Plate Technique (RBPT), Enzyme-linked immunosorbent test (ELISA) for detection of IgA, IgM and also milk ring test to detect the antibodies of brucella agent. Also, the samples subjected by Real Time PCR to determine the presence of bcsp31 gene (Brucella gen). Out of the total examined samples (133) 18.26%, (139) 19.09%, (3) 0.41% and (126) 17.3% were tested positive by RBPT, ELISA IgG, IgM and Real Time PCR respectively. specificity and Sensitivity of RBPT compared with real- time PCR were 98.9% and 79.59% respectively. While, sensitivity and specificity of ELISA for IgG with Real Time PCR were 79.59% and 100% respectively, while, for ELISA IgM were 6.12% and 100% respectively. In conclusion, we suggest using Real Time PCR as a supplementary test in the diagnosis of brucella disease and as a confirmatory test in suspicious cases.

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INTRODUCTION

Brucella disease is considered the main bacterial infection causes abortion in animals. It caused by Brucella species which has a public health hazard as it considered of the most important animal origin diseases. Brucellosis is endemic in Mediterranean and Middle East regions [1,2]. It is responsible for high economic losses as it causes abortion, decrease in milk production, animals' infertility as well as money losses in animal treatment [3,4].

Early detection of brucellosis may lower effort and time in animal treatment. It is very important to determine brucellosis incidence rate to eliminate and control the diseases. There are different methods used for diagnosis of brucellosis which help in eradication and disease control including, bacteriological examination, serological diagnosis and molecular techniques. Bacteriological diagnosis lacks sensitivity, and not suitable in huge eradication system's [3,4]. Conversely, Serological examination is more sensitive and easily to apply in diagnosis of brucellosis in huge eradication program comparing to bacterial culture [5]. Rose Bengal Plate Technique (RBPT)- test and Enzyme-linked immunosorbent test (ELISA) are certified by previous study [6] for diagnosis of brucellosis on a large scale. RBPT usually considered as a screening method for brucellosis but ELISA technique considered as a diagnostic method. Both

tests are depending on antigen antibody reaction [7]. Despite the practical availability and sensitivity of serological tests it has limitations because of false positive reaction may developed due to Cross-reactivity between other gram-negative bacteria [7].

Molecular assays, such as real-time (rt) PCR were recently used successfully for diagnosis of brucellosis in certain laboratories [8]. Hence, the goal of this research is to help identify what proportion of cases could be possibly lost in routine handle of antigen -antibody reaction (RBPT and ELISA assay) and to situate the role of Real- Time PCR in improving the case diagnosis and removal of brucellosis in Libya.

METHODS

Collection of Samples

Overall, 1456 samples (blood and milk) were collected from 728 sheep and goats' brucellosis suspected brought to the Veterinary Clinical Complex from areas in and around Al Bayda, Al Jabal Al Akhdar, in east of Libya from November 2020 to June 2021. The samples of blood were carefully drawn from the jugular vein into vacuum tubes without EDTA (Anticoagulant) for serum separation by vacationer system and transported directly to laboratory of Animal Health Department of the Ministry of Agriculture, Libya for serological and molecular examination.

RBPT

All the sera were tested by RBPT for diagnosis the antibodies of brucella. The method was conducted according to the recommended procedure by previous study [9] using antigen obtained from Institute Pourquier, France. The results were interpretation by the degree of agglutination [10].

ELISA

The steps of ELISA and interpreted the results was done according to the directions of the manufactures using kit of IgG ELISA test (ID VET, France). The Brucella LPS coated all the wells of the Microplate; Individual sera were diluted and incubated in the wells. Brucella antibodies whether meltiness or abortus found in the serum will form complex between LPS and antibody immune that stay connected in the well. For detection of IgM, ELISA Brucella IgM test kit (Immunospec –USA) was used. Reading and interpretation of results were done following the instruction of manufacturer.

Milk Ring Test

Because the sheep and goats' milk are less of fat than the cows, milk, the positive reactions are than manifested by agglutination of the antigen which usually falls to the bottom of the tube leaving the milk column white, however if the density of blue color in the skim portion is lower or less than in the fat layer, the result is considered positive. If the density of blue color in the fat layer is equal to or less than of the skim portion, the test is considered negative.

Real -Time PCR technique

RT- PCR was performed to amplify bcsp31 gene encoding Brucella surface protein using Brucella Real Time PCR Kit (LiferiverTm, Shanghai, China) according to the manufacture instructions. The kit contains DNA Extraction Buffer, Brucella Reaction Mix, PCR Enzyme Mix, internal control and Brucella Positive Control (1×107 copies/ml). DNA was extracted from blood samples using extraction buffer supplied in the kit following the instructions of the manufacturer. The primers and probes of PCR that used in this research were selected according to earlier study [11].

Cycling conditions include 37 o^c for 2 min, 94 o^C for 2 min then 40 cycles of 93 o^C for 15sec and 60 o^c for 1min. The reactions were performed in an Applied Biosystems Real Time PCR machine. A fluorescent dye (sybr® green I) binds directly to DNA (dsDNA) the dye produces a signal that is proportional to the DNA concentration. An external positive control (1×107 copies/ml) available with the kit was used. All samples were tested in duplicate; the results were interpreted following the manufacture instructions, cycle threshold (CT) values ≤38 cycles were interpreted as positive. The values between 38〜40 was Re-tested; if it is still 38~40, it was reported as negative.

Statistical analysis

The overall agreement, specificity and sensitivity between the various diagnostic method were calculated as per standard procedures using methods described by pervious study [12].

RESULTS AND DISCUSSION

By serological testing of 728 serum samples and the same number milk samples collected from sheep and goat, (139)19.09%, (3) 0.41%, (133)18.26%, (126)17.3%, were tested positive by ELISA IgG, ELISA IgM, RBPT and MRT respectively. While, 143 (19.64%) The positive sera were retested for detection of Brucella gene by using R.T-PCR (Table 1).

Table 1. Brucella-antibodies and/or Brucella-specific DNA

Tests	Samples No		Positive		Percentage		Total Positive			Total Sheep & Goat %
	Sheep	Goat	Sheep	Goat	Sheep	Goat	Both	Sheep	Goat	
ELISA IgG	324	404	49	90	15.1%	22.3%	139	35.3%	64.7%	19.09%
ELISA IgM	324	404	0	3	0%	0.7%	3	0%	100%	0.41%
RBPT	324	404	45	88	13.9%	21.8%	133	33.8	66.2	18.26%
MRT	324	404	35	91	10.8%	22.5%	126	27.8	72.2	17.3%
RT-PCR	324	404	50	93	15.4%	23.0%	143	35%	65.03	19.64%

In comparison to our results, an earlier study [13] recorded a lower detection rate with real time PCR (13.6%) compared with RBPT (15.6%) and in accordance with our findings, they reported that real time successfully for diagnoses of Brucella in 11.1% of the sero-negative samples and they related this to the probability of the presence low level of antibodies in cases of acute infection or chronically infected animals. Additionally, another study also reported that 36.96 % sero-negative samples tested by RBPT and ELISA gave positive by real-time PCR [11].

In the current study, forty-two samples were tested positive by ELISA IgG and IgM. These findings indicated that ELISA gave a more accurate result of true positive when both methods are used together in diagnosis of brucellosis. While, RBPT has six false positive samples and six false negative samples. This could be suggestive of the event when the molecular technique is not obtainable, the ELISA method can be used. These results are in agreement with previous report[14] who confirm that ELISA is a beneficial assay in detection of brucellosis.

Serological tests depending on the formation antigen antibody complex, brucella antigen gets into a target cell and stimulate the immune system to produce antibodies, therefore, is being easily detected by serological method but it needs some time to happen. By using serological tests may occur false negative results due to the delay in the production of antibodies [15]. Also, animals with sub-acute disease cannot be easily differentiated with healthy animals by serological methods. In contrary, R.T- PCR detected the DNA in the infected cell.

The results of antigen antibody's reaction when comparison with R- T- PCR were shown in table 2 and as it shown, the sensitivity and specificity of RBPT in comparison with R- T PCR were 79.59% and 98.91% with an overall agreement of 97.3%. The specificity and sensitivity of ELISA IgG with R.T- PCR were 100% and 79.59 % with an overall agreement of 98.3%, in case of ELISA IgM the sensitivity and specificity were 6.12% and 100% with overall agreement of 98.3% with R- T PCR. The negative- predictive values was higher for ELISA IgM or IgG indicate that these tests were highly specific but ELISA IgM has a lower sensitivity than RBPT.

Sensitivity and specificity indicate that ELISA IgG is acceptable in diagnosing of brucellosis, therefore, it best substitutions in cases where real-time PCR is not available. However, the sensitivity and specificity of RBT and ELISA IgM respectively confirm that the antigen antibody's reaction are weak in the detection of Brucella agent compared to real- time PCR. These results agreed with [16] who found that 100% sensitivity and 98.3% specificity of RT- PCR compared with a blood culture. In addition, another study reported that RT- PCR has a more specificity and sensitivity than sociological method and is capable of detecting low levels of Brucella gene [17]. Therefore, it is a useful assay in confirming Brucella infection [18]. On the other hand, there is a limitation of real- time PCR because it needs high experience and it is very expensive so it is not easy to be routinely used especially in developing countries, thus, some level of emphasis should be placed on it at least at referral laboratories to help improve case detection.

Milk samples result confirmed the presence of Brucella-antibodies in 91 goats milk sample, while in 35 samples, sheep. In 324 and 404 from sheep and goats, respectively, confirmed with Brucella-specific DNA positive were detected in serum samples. As the table (2) found that 88% sensitivity and 100% specificity of MRT according to real-time PCR assay for Brucella agent. Table (2) noted that the number of positive samples in the goats by the milk ring test (91) relatively close to goats result in the RT- PCR (93) while the number of positive samples in the sheep by the milk ring

test (35) that very different to Goats result were (91) this variation maybe because the sheep milk is less of fat than the goat's milk (Figure 1).

Specificity of M. RT, ELISA IgG, ELISA IgM were very high (100%) but the specificity of RBPT was lower and sensitivity of this tests were 88.1%, 79.59%, 6.12% respectively. the RBPT more sensitive than ELISA IgM 79.59%, 6.12% respectively. On the other hand, ELISA IgM more specialty than RBPT.

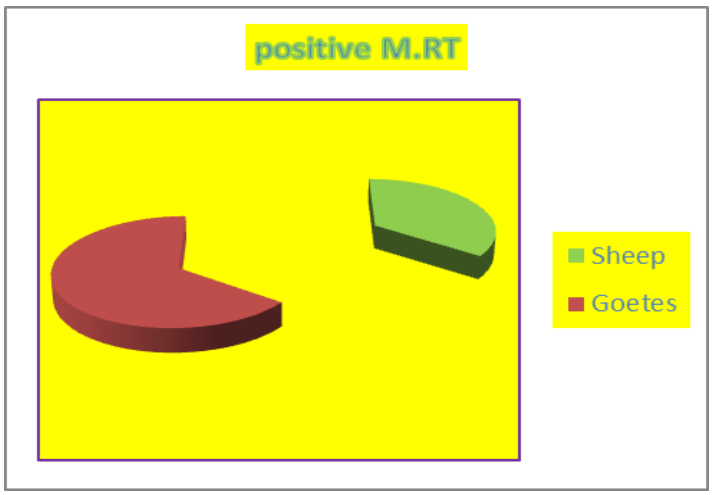

Figure 1. Positive Results of sheep and Goats by MRT

Table 2. Results of Serological tests versus Real- time PCR

Category I: Milk Ring Test (M.RT) versus Real time PCR		**No. of samples**	**Sens**	**Spec**	**P.V.P.T.R**	**P.V.N.T.R**	**Overall agreement**
M.RT	R.T-PCR						
Positive	Positive	126	88.1%	100%	100%	95.5%	96.3%
Negative	Negative	575					
Positive	Negative	0					
Negative	Positive	27					
Category II: Rose bengal Agglutination Test (RBPT) versus R.T-PCR		No. of samples	Sen	Spe	P.V.P.T.R	P.V.N.T.R	Overall agreement
RBPT	R.T-PCR						
Positive	Positive	133	79.59%	98.91%	86.66%	98.19 %	97.3%
Negative	Negative	577					
Positive	Negative	8					
Negative	Positive	10					
Category III: ELISA IgG versus Real time PCR		No. of samples	Sen	Spe	P.V.P.T.R	P.V.N.T.R	Overall agreement
ELISA	R.T-PCR						
Positive	Positive	139	79.59%	100%	100%	98.92%	98.3%
Negative	Negative	585					
Positive	Negative	0					
Negative	Positive	4					
Category IV: ELISA IgM versus Real time PCR		No. of samples	Sen	Spe	P.V.P.T.R	P.V.N.T.R	Overall agreement
ELISA	PCR						
Positive	Positive	3	6.12%	100%	100%	92.29%	98.3%
Negative	Negative	585					
Positive	Negative	0					
Negative	Positive	140					
Total of blood samples tested		728					

P.V.P.T.R= predictive value positive test result, P.V.N.T.R= predictive value negative test result, Sens=sensitivity, Spec=specificity

CONCLUSION

In this study the presence of false negative results with both RBPT and ELISA and the limitation of serological tests in the detection of brucella disease in the sub-acute stage of disease suggested that samples should be retested by either culture or molecular techniques. In addition, our findings confirm superiority of real-time PCR in detection of brucellosis.

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Conflict of interest. Nil

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التشخيص الجزيئي وأنواع من اختبارات الاجسام المضادة للكشف عن مرض البروسيلا في الحيوانات المجهضة والسليمة ظاهريا في الاغنام والماعز في وحول مدينة البيضاء ليبيا

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المستخلص

الهدف من هذه الدراسة هو تقييم الاختبارات التي تعتمد علي التفاعل بين الاجسام المضادة و الانتجين مقارنة بجهاز تفاعل البلمرة المتسلسل ذو الوقت الحقيقي لتشخيص مرض البروسيلا في الاغنام والماعز. لذلك سبعة مائة وثمانية وعشرون عينة دم تم جمعها من حيوانات سليمة ظاهريا وكذلك نفس العدد عينات حليب سحبت من نفس الحيوانات. المشتبه أصابتها بالبروسيلا والمشخصة من العيادات البيطرية في وحول منطقة البيضاء. ليبيا. عينات الدم تم اختبارها بواسطة الاختبارات السيرولوجيه والتي تشمل اختبار لوحة بنقال, الاليزا IgG, IgM وكذلك اختبار حلقة الحليب للكشف عن وجود الاجسام المضادة للبروسيلا. وكل عينات الدم تم اعادة فحصها بواسطة تفاعل البلمرة المتسلسل ذو الوقت الحقيقي لتحديد وجود جين bcsp31(جين البروسيلا). وكانت النتائج كالتالي 18.26%(133, 19.09%(139), 0.41% (3) و17.3%(126) علي التوالي. خصوصيه وحساسية اختبار لوحة بنقال مقارنة بتفاعل البلمرة المتسلسل ذو الوقت الحقيقي كانت 79.59% ,98.9% علي التوالي في حين حساسية و خصوصيه الاليزا IgG مع بتفاعل البلمرة المتسلسل ذو الوقت الحقيقي 79.59% و100% علي التوالي اما الاليزا IgM فكانت 6.12% و100% علي التوالي. الخلاصة نقترح استخدام تفاعل البلمرة المتسلسل ذو الوقت الحقيقي في تشخيص مرض البروسيلا كاختبار تأكيدي للحالات المشتبه بها.

الكلمات الدالة. داء البروسيلات، الأغنام، الماعز، علم الأمصال، تفاعل البوليميراز المتسلسل في الوقت الحقيقي.