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RESEARCH ARTICLE

Diagnosis of Brucellosis in Ruminants in Kafr El-Sheikh governorate, Egypt

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Abstract

Brucelosis is one of the most important diseases affecting animals in most of the developing countries. The study aimed to serological detection of Brucellosis in ruminants in Kafr El-Sheikh governorate using RBPT and BPAT as a screening test and ELISA and CFT as a confirmatory test. The obtained result showed that the incidence rate was highest in sheep (12%) followed by cattle (8.4%), goat (6.4%) and finally the lowest incidence rate was in buffaloes (6.1%). The most infected area was Fuwwa and kafr El-sheikh in comparison with other districts. Moreover, iELISA is most sensitive (96.5%) and specific test (98.3%) while BAPT is the lowest sensitive (83.8%) and specific test (90%). The isolated Brucella strain bacteriologically identified and confirmed by PCR as *B. meltensis* biovar 3.

INTRODUCTION

Brucellosis is a contagious bacterial infection of livestock and continues to be of great health concern and economic importance worldwide, especially in Mediterranean countries (Munir et al., 2010). The disease is caused by aerobic, Gram-negative bacteria of the genus Brucella. The disease causing abortions, fetal death, genital infections and sterility (Probert et al., 2004). The transmission of the disease is by direct or indirect contact with infective execrators. They are shed in large numbers in the animal urine, milk, placental and other fluids (Otlu et al., 2006; Zvizdic et al., 2006).

Among the different species of genus Brucella, *B. aboruts* is the common strain infecting cattle all over the world while *B. melitensis* is affecting mainly sheep, goats and also other species (Alton, 1990). Cattle Brucellosis is usually caused by *B. abortus* biovars and occasionally by *B. melitensis* (OIE, 2008). In Egypt, starting from 1998 *B. melitensis* was reported to be the common strain isolated from cattle as reported by Shalaby et al., (2003).

Diagnosis of Brucellosis is based on isolation of the organism from infected animals but this is a cumbersome and time consuming task, due to the fact that these fastidious organisms grow slowly on primary isolation (Meyer, 1981). Moreover, it is not possible to isolate Brucella every time, even from infected individuals. Therefore, assessment of antibody response employing serological test plays a major role in the routine diagnosis of Brucellosis and supported where appropriate by bacteriological examination (Alton et al., 1988).

The routine identification and differentiation of Brucella species is based on phenotypic traits, but it is associated with a high risk of laboratory acquired infections and is very time-consuming (Navarro et al., 2004; Carver et al., 2005). Therefore, the molecular diagnostic techniques such as real-time PCR is more preferable that are simpler, faster, and less hazardous. At present, there are several conventional and real-time PCR assays for differentiation between Brucella species (Foster et al., 2008; Reisberg et al., 2013).

The purpose of this study was to evaluate the most commonly used serological tests, including rose Bengal plate test (RBPT), buffered acidified plate test (BAPT), Indirect ELISA and complement fixation test (CFT). In addition to, identification of Brucella strains by PCR and estimation of sensitivity, specificity and ability of applied tests.

1. Material and Methods

2.1 Samples

A total of 1450 serum samples were collected from cattle, buffaloes, sheep and goat from different districts in kafr El-Sheikh province. All serum samples kept at 20 ^{o}C for serological examination.

A total of 54 tissue specimens were collected from some reactors for bacteriological examination. Lymph nodes, especially supramammary and retropharyngeal lymph nodes were taken from the carcasses, including the surrounding fat and without cutting of the obtained lymph nodes. The collected samples were packed in sterile disposable plastic bags and were transferred on ice packs to the Brucella department laboratory, Animal Health Research Institute, Dokki, Egypt as soon as possible. They were kept frozen at -20 ^{o}C until cultured.

2.2 Bacteriological examination

Bacterial culture for the different tissue specimens, aborted materials of reactor animals to one or more of the serological tests was carried out on tryptic soy agar containing antibiotics. The suspected Brucella colonies were subjected to further identification by morphological characters of the colonies and microscopically examination of a smear made from suspected growth stained by Gram's and Modified Ziehl-Neelsen stains. The identification of Brucella species was carried out according to the method recommended by the Alton et al., (1988).

2.3 Serological examinations

All serum samples were examined for antibodies against Brucella with RBPT and iELISA according to Morgan et al., (1978) and BAPAT and CFT according to Alton et al., (1988).

2.4 Detection of Brucella-DNA by PCR

a. Preparation of killed bacteria for PCR

When bacterial cells were used directly for PCR, All bacteria were killed by the addition of 67% methanol-33% saline. The killed bacteria were rinsed one time in distilled water to remove the methanol and were then resuspended in distilled water at an optical density of 0.15 to 0.20 at 600 nm (approximately 10^9 cells per ml).

b. Preparation of genomic DNA

DNA was isolated from Brucella isolate as described previously (Halling et al., 1991). Briefly, cells were incubated at 50°C for an hour in detergent solution (Zwitter-agent 3-14, 1%) containing 0.1 M citric acid. The cells were washed in TE (10 mM Tris-HCl, 1 mM EDTA {pH 8.0} and then lysed by the sequential addition of lysosome, proteinase K, DNA was extracted with phenol and precipitated with ethanol. The DNA dissolved in TE and kept at -20^{o}C till PCR examination.

c. PCR assay

The PCR assay for Brucella spp. targeting IS711 gene was performed according to Bricker and Halling (1994) using a specific set of primers which synthesized by (MWG Biotech, Berlin, Germany) as shown in table 1.

The PCR assay was carried in 25 µl reaction volume, master mix contained 1 µl from each primer (10 pmol/ µl), 4.5 µl RNase-free water and 12.5 µl of Quantitect MasterMix (Qiagen, Hilden, Germany). Finally 5.0 µl template was added.

The amplification was performed in thermocycler mastercycler (Eppendorf, Wesseling-Berzdorf, Germany) using the following condition: a preliminary heating for 15 min at 95°C, 35 cycles of 60 Sec at 94°C, 45 Sec at 60°C, 60 Sec at 72°C and the final elongation of 10 min at 72°C. The PCR products were detected through 1.5 % agarose gel stained with etidium bromide solution (0.5 mg/ml) and visualized under an ultraviolet transilluminator and photographed.

2. Results

3.1 Serological results

The collected serum samples represented different areas at Kafr El-Sheikh governorate were examined using screening tests as (RBPT and BAPAT) and other serological confirmatory tests as (iELISA and CFT). The results of serological examinations were differed, the RBPT detected more reactors (130) than BAPAT (119), CFT (122) and iELISA (125) as shown in table 2. Therefore, the RBPT considered a good field screening test.

Consequently, the incidence rate of Brucellosis based on RBPT showed high percent of positive reactors in Fuwwa (14.4%) followed by Kafr El-Sheikh (13.5%) and Bieyala (11.8%) than other areas of Kafr El-Sheikh governorate as shown in table 3.

The percent of positive reactors obtained by RBPT as a screening test ranged between 6.1-12 %. The overall Brucellosis incidence rate was highest in sheep (12%) followed by cattle (8.4%), goat (6.4%) and finally the lowest incidence rate was in buffaloes (6.1%) as shown in table 4.

Statistical analysis of the obtained results with different serological tests showed that the iELISA is most sensitive (96.5%) and specific test (98.3%). In contrast, the BAPT is lowest sensitive (83.8%) and specific test (90%) in comparison with other examined tests as shown in table 5.

2.2 Result of bacteriological examination

Successful isolation of *B. melitensis* from collecting materials from emergency slaughter reactor animals. All isolates of *B. melitensis* were identified as *B. melitensis* biovar 3.

3.3 Results of PCR assay

The obtained isolates in this study were examined with multiplex conventional PCR assay using specific primer pairs for *B. abortus* and *B. melitensis*. The result of PCR amplification showed that 731 bp fragment which atypical for *B. melitensis* as shown in figure 1.

Table (1): Oligonucleotide primers used for Brucella DNA amplification

Primer code	Primer sequences	Product size	Species specificity
IS711-SP	5`-TGCCGATCACTTAAGGGCCTCAT-3`	498 bp	*B. Abortus* (biotype
Ba-sp	5`-GACGAACGGAATTTTTCCAATCCC-`3		1, 2 & 3)
Bm-sp	5`-AAATCGCGTCCTTGCTGGTCTGA-3`	731 bp	*B. melitensis*

Table (2): Results of different serological tests for Brucellosis in different areas in Kafr El-sheikh governorate

Area	No. of Examined Animals	Number of positive reactors RBPT	BAPAT	CFT	iELISA
Kafr El-Sheikh	260	35	33	33	34
Bieyala	220	26	24	24	25
Disuq	183	8	7	7	8
Fuwwa	180	26	25	25	25
Sidi Salim	170	12	9	10	10
Motobas	122	5	5	5	5
Qillin	170	14	13	14	14
El-Hamool	145	4	3	4	4
Total	**1450**	**130**	**119**	**122**	**125**

Table (3): The incidence rate of Brucellosis among different animals at different areas of Kafr El-Sheikh governorate using Rose Bengal Test.

Region	Total animal	RBPT			
		Reactors	%	Non-Reactors	%
Kafr El-Sheikh	**260**	35	13.5	225	86.5
Bieyala	**220**	26	11.8	194	88.2
Disuq	**183**	8	4.4	175	95.6
Fuwwa	**180**	26	14.4	154	85.6
Sidi Salim	**170**	12	7.1	158	92.9
Motobas	**122**	5	4.1	117	95.9
Qillin	**170**	14	8.2	156	91.8
El-Hamool	**145**	4	2.8	141	97.2

Table (4): Results of different serological tests for Brucellosis in different animals species

Animal species	Examined Animals	RBPT		BAPAT		CFT		iELISA	
		+ve	%	+ve	%	+ve	%	+ve	%
Cattle	**545**	46	8.4	41	7.5	43	7.9	45	8.3
Buffaloes	**280**	17	6.1	13	4.6	15	5.4	16	5.7
Sheep	**475**	57	12	56	11.9	55	11.6	55	11.6
Goats	**150**	10	6.6	9	6	9	6	9	6

Table (5): Estimation of Sensitivity and specificity of different serological tests

Diagnostic sensitivity/specificity	Percentages of serological tests			
	RBPT	BAPAT	CFT	iELISA
Sensitivity	93	**83.8**	92	**96.5**
Specificity	91.9	**90**	97.2	**98.3**

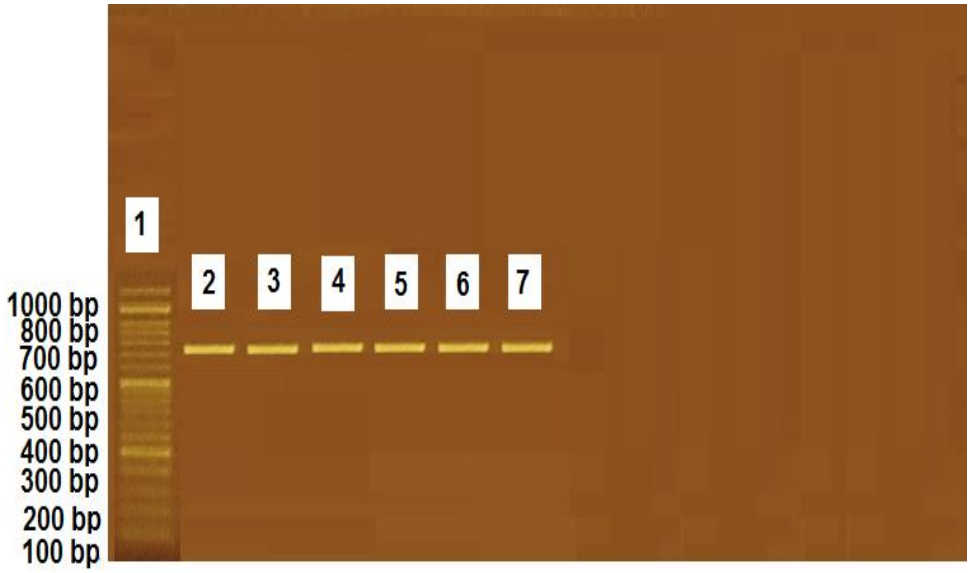

Figure (1): PCR amplification products of IS711 gene for Brucella. The figure showed; Lane 1: DNA ladder and lanes 2-7: *B. melitensis* biovar 3 field strains

3. Discussion

Rapid and accurate diagnosis is fundamental for control and eradication of Brucellosis (Refai, 2003). Culture provides the definitive diagnosis of Brucellosis and is considered the gold standard method for it (Alton et al., 1988). Because of difficulty of performing culture in the field, its consuming time, its health hazard and lack sensitivity of the most culture procedures. So, the diagnosis of Brucellosis is mainly depends upon the application of different serological procedures which still play the major role in the routine diagnosis of Brucellosis among animals (Yu and Nielsen, 2010).

The results of RBPT revealed that the highest number of reactors 130 (9%) followed by BAPAT 119 (8.2%), CFT 122 (8.4%) and IELISA 125 (8.6%), respectively. The obtained result indicates that the RBPT is the most accurate indicator and initial screening for Brucellosis (Nicoleltti, 1980). A similar conclusion was also reported by Ammar (1995) and Abou Baker et al., (2010).

In addition, the results from this study indicate that bovine Brucellosis is more prevalent in Fuwwa (14.4%) followed by Kafr El-Sheikh (13.5%) than other examined districts. The difference in infection rates between different districts in Kafr El-Sheikh governorate may be due to difference in applied management in each area or failure or absence of vaccination program in some herds.

The overall Brucellosis incidence rate was highest in sheep (12%) followed by cattle (8.4%), goat (6.4%) and finally the lowest incidence rate was in buffaloes (6.1%) depending on the result obtained with RBPT.

The obtained results agree with previous finding by Shalaby et al., (2003) where the percent of positive reactors was 8.4%, 5.82%, 9.82% and 5.6% in cattle, buffaloes, sheep and goat, respectively.

The rates of Brucella infection among cattle was higher than rate estimated by Mahmoud (1991) reported that (2.2%) in cattle and lower than other as El-Gamal (2004) reported that (12%) in cattle. While the obtained rate in buffaloes is higher than the previous reported rate 3.09%, 0.35% by Mahmoud (1991) and Montasser et al., (2002), respectively. The obtained results are high in comparison with result of previously reported rate in sheep as 4.73% by Mahmoud (1991), 3.5% by Montasser et al., (2002) While it come in accordance with Hagazy et al., (2011) who reported reactors percent 12.2% among sheep.

Differences between the prevalence of Brucellosis obtained in this study and those obtained by other authors may be attributed to various factors such as the year during which this study was performed, the area from which animals were examined, as well as the evolutionary changes in the animal husbandry which affect the rate of exposure and the different serological tests used confirmed by bacterial isolation and PCR or not.

This study used to evaluate the diagnostic performance of iELISA test in comparison with other serological tests (RBPT, BAPAT and CFT). The average sensitivity (96.5%) and specificity (98.3%) of iELISA among different animal species reported to be high in comparison with other serological tests as shown in table 5. This finding agrees with results obtained by (El-sharakawy, 2004; Abernethy et al., 2012).

Its superior sensitivity and specificity of iELISA may attributed to, it is not affected by anti-complementary activity and detects both IgG1**,** IgG2 and other subclasses of immunoglobulin that indicated in early and chronic Brucella infection. Also the sensitivity of BAPAT is low that attributed to acidic pH (4.0) of its antigen (OIE, 2008).

The identification of the obtained isolate revealed that *B. melitensis* biovar 3 is the sero type existed in examined animals in Kafr El-Sheikh governorate. Similar findings were reported by many authors as Ammar, (2000), Montasser et al., (2001) and Hagazy et al., (2011).

The obtained Brucella isolates from positive reactors animals were examined by multiplex conventional PCR for detection and identification of *B. abortus* and *B. melitensis.* The obtained results revealed amplification only with *B. melitensis*.

The PCR is a highly sensitive method which makes it possible to detect nucleic acid amplification products. The results can be obtained rapidly so that they can be used not only to support bacteriological investigation, but also to make them more reliable (Gallien et al., 1998). In addition, the PCR assay can simultaneously detect and differentiate of *B. abortus* and *B. melitensis* in the same time and one reaction (Mirnejad et al., 2012).

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