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SEROLOGICAL AND MOLECULAR DIFFERENTIATION BETWEEN *BRUCELLA* AND *YERSINIA ENTEROCOLITICA* O:9 INFECTIONS IN CATTLE

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ABSTRACT

Brucellosis is one of the world's major zoonoses that still has veterinary, public health and economic concern in many parts of the world. In livestock, brucellosis is the major impediment for trade and export. In this work a total of 118 different samples (45 serum samples, 45 blood samples, stomach contents of 18 aborted feati and 10 fecal samples) were collected from 45 diseased and apparently healthy cows at selected veterinary clinics at El-Sharkia governorate. The serum samples were subjected to investigation by different serological methods (Rose Bengal, BAPAT, CFT and ELISA) for detection of bovine brucellosis. While, molecular differentiation between *Brucella* and *Yersenia enterocolitica* O:9 were made on blood samples, stomach contents and fecal samples of sero positive animals for brucellosis using multiplex PCR assay. The results revealed that 26 out of 45 serum samples were positive for brucellosis, as detected by ELISA while 19 samples gave positive amplification of 223bp for *Brucella* and seven positive amplification of 325bp for *Yersinia* representing 26.92% of total brucellosis seropositives of which two samples (7.69%) were positive for both Yersinia and Brucella as tested by multiplex PCR assay. Therefore, we concluded that multiplex PCR proved to be a reliable molecular method for differentiation between *Brucella* and *Yersinia* as rapid diagnostic tool directly from clinical specimens.

***Key words**: Multiplex PCR, Brucella, Yersinia enterocoliticaO:9, Serological investigation.*

INTRODUCTION

Brucellae are gram-negative facultative intracellular bacteria causing brucellosis, which remains a zoonosis of worldwide public health and economic importance (Marcin *et al.*, 2012). The economic impact of brucellosis in animals can be devastating (Gorvel 2008), especially in developing countries (Erika *et al*., 2012).

Y. enterocolitica serotype O:9 is one of the human pathogenic serotypes, which can also infect animals, generally without causing any symptoms. Pigs, sheep, and cattle can be carriers of pathogenic serotypes of *Y. enterocolitica* in their intestinal flora. Prevalence of *Y. enterocolitica* seems to be on the rise, at least in cattle, as it was rarely seen before the 1990 and since then has been regularly isolated (Erika *et al.*, 2012).

Brucella, however, is a slow growing organism and cultures are rarely positive before the fourth day of incubation. Usually cultures become positive between the first and third week, and should be kept for at least 45 days before the culture can be concluded to be negative for *Brucella* (Henk and Manzoor 2005).

Moreover, the conventional isolation and identification procedures for the detection of *Y. enterocolitica* clinical samples are time consuming taking at least 2-4 weeks owing to the cold enrichment procedures being followed and require several phenotypic assays for the differentiation of pathogenic and non-pathogenic yersiniae (Kapperud 1991; Lübeck *et al.*, 2003 and Balakrishna *et al.*, 2012).

Although, many cross-reacting microorganisms may yield false positive results for Bovine brucellosis, only *Yersinia enterocolitica* O:9 is a significant cause of false-positive serological reactions (FPSR) in the diagnosis of bovine brucellosis (Gerbier *et al.*, 1997). The gradual increase during 1990 in herds infected with *Yersinia enterocolitica* serotype O:9 has created an international problem in laboratory diagnosis of brucellosis (Cheasty *et al.*, 1998; Lübeck *et al.*, 2003).

Eradication of brucellosis is based on the serological testing of animals and the subsequent culling of those that are seropositive for antibodies to *Brucella*. Thus, the specificity of the serological tests used is of paramount importance, (Raúl *et al*., 2005) and the

need for accurate reliable diagnostic methods becomes important.

PCR technique could be a potentially useful method for the diagnosis of brucellosis since it could detect the bacteria in samples even if highly contaminated with other microorganisms. In addition, PCR technique could detect more infected animals compared to serological methods (Romero and Lopez-Goni 1999; Leal-Klevezas *et al*., 2000; Cortez *et al.,* 2001 and Ayman and Nermeen 2010).

The aim of the current study was to use multiplex PCR technique for simultaneous accurate detection and differentiation of *Brucella* and *Yersinia enterocolitica* O:9 to avoid false culling of seropositive animals and subsequent economic losses.

MATERIALS and METHODS

Animals: A total of 45 diseased and apparently healthy cows collected from some veterinary clinics at El-Sharkia governorate were used for this work.

Sampling:
A total of 118 different samples were collected. The samples were classified as follow (45 serum samples, 45 blood samples, stomach contents of 18 aborted feati and 10 fecal samples) the detailed data reported in Table (1). Serum samples were collected in clean dry centrifuge tubes without anticoagulant, left to clot, centrifuged at 1500xg for 20 minutes for serum separation used for serological investigation kept at -20°C until analyzed. Blood samples was collected on EDTA as anticoagulant used in PCR assay.

Bacterial strains:
Brucella abortus strain 19 and *Yersinia enterocolitica* O:9 were kindly provided by the Brucella Department Animal Health Research institute, Dokki, Giza used as control positive for PCR assay.

I- Serological investigation:

1. Buffered acidified plat antigen test (BAPAT): The test was applied according to (Anon 1984) where, any degree of agglutination within 8 minutes (≥ 20 IU $ml.^{-1}$) was considered positive and if no agglutination within 8 minutes was regarded as negative**.**

2. Rose-Bengal plate test (RBPT): The test was conducted according to (Alton *et al*., 1988) where, any degree of agglutination within 4 minutes (≥ 25 IU ml^{-1}) was considered positive and if no agglutination within 4 minutes was regarded as negative.

3. Complement Fixation test (CFT): The test was performed according to (Ibrahim 1996) using G.Pig serum as a source of complement and a pretitrated amount of 2% sensitized sheep erythrocytes.

4. Indirect Enzyme Linked Immunosorbant Assay (ELISA): The test was applied according to (Alton *et al.*, 1988). Briefly by using *Brucella* LPS as coating antigen and the tested sera diluted in 1/100 PBS (pH 7.2) were added. The plates incubated at 37˚C for 30 minutes. After washing 200ul of conjugate were added then 200ul of freshly prepared OPD were added. Finally the plates were read spectrophotometrically at 492nm.

II- Molecular investigation Multiplex Polymerase chain reaction:

Extraction of crude DNA from bacteria: DNA template was prepared by boiling according to (OIE 2009) for brucella and according to (Balakrishna *et al.,* 2012) for *Yersinia enterocoltica* O:9 finally centrifuged for 10 min and the supernatants were used as DNA templates for PCR amplification stored at -20 till use.

DNA extraction from stomach contents of aborted faeti: The procedure was applied according to (Cetinkaya *et al.,* 1999). 300ul of stomach contents suspended in 300 ul distilled water were incubated at 56°C for 45 minutes. 100ul of 2 % SDS and IM sodium hydroxide mixture and 50 ul PBS (pH 7.4) were added to the suspension. The suspension was vortexed and boiled for 30 minutes and then left to cool. 100 ul of 1M Tris-HCl (pH 6. 8) was added to the suspension and it was again shaken vigorously and centrifuged at 11,600g for 15 minutes. 300 ul of the supernatant was transferred to another tube. DNA was purified by successive phenol, phenol-chloroform-isoamyl alcohol (25:24:1), and chloroform-isoamyl alcohol extractions followed by precipitation with ethanol overnight at -20°C. The DNA was dissolved in 100 uL of distilled water according to Maniatis *et al*. (1982).

The DNA purification from feces: was based on procedures developed by Gumerlock *et al.* (1991) and by Collins *et al.* (1993). Briefly, one gram of feces, was suspended in 10 ml of phosphate buffered saline (pH 7.4) and shaken for 5 min, and sedimented for 30 min. One milliliter of supernatant was centrifuged at 13,000 3 *g*. The pellet was resuspended in 670 ml of TE-sucrose (50 mM Tris-HCl [pH 8] 50 mM EDTA, 20% sucrose), 300 ml of SDS (10%), and 30 ml of proteinase K (20 mg/ml). The mixture was incubated at 50C for 60 min. DNA was extracted with phenol: chloroform and then precipitated with ice-cold ethanol; the DNA pellet was then dissolved in 50 ml distilled water.

Extraction of DNA from blood samples: DNA was extracted from bovine blood samples according to Mukherjee *et al.* (2007) using a slight modification of a protocol published by Leal-Klevezas *et al.* (1995).

Multiplex PCR: PCR reaction was performed in a total volume of 25 µl with 5 µl of the DNA template, 25 pmol of each oligonucleotide primer (Metabion international AG), of both brucella and yersinia detailed sequence of each primer shown in table (2), 12.5ul of 2X master mix and nuclease free water up to 25ul. The cycling protocol listed in Table (3).

The primers B4 and B5 were designed to amplify a target sequence of 223-bp within a gene of Brucella cell surface protein (BCSP) code for the production of a 31-kDa membrane protein specific to the genus *Brucella*. Also, primer used to amplify a target sequence of 325-bp for *Yersinia* specific region of the 16S rRNA gene has been used to detect *Yersinia* spp.

Analysis of PCR products:
The analysis was carried out according to Sambrook *et al.* (1989) using 1.5% ethidium bromide stained agarose gel and visualized under ultraviolet transilluminator.

RESULTS

Results of serum samples investigated by different serological methods (Rose Bengal, BAPAT, CFT and ELISA) for detection of bovine brucellosis reported in Tables (4) and (5) where the highest positive number of samples for brucella was obtained by BAPAT test (31samples out of 45 serum samples). On the other hand, RBPT test showed the lowest positive samples (23 samples out of 45 serum samples).

A number of seventy three samples (45 blood samples, 18 stomach contents of aborted fetii and 10 fecal samples) were subjected to investigation by multiplex PCR for amplification of Brucella cell surface protein (BCSP) &16S rRNA genes of both *Brucella* & *Yersenia* respectively. Results for positive amplification of expected amplicons 223-bp and 325-bp of both *Brucella* and *Yersenia enterocolitica* O:9 are shown in Fig (1).

The results of multiplex PCR are presented in table (6) where out of 45 blood samples 19 samples gave positive amplification for *Brucella*, five samples gave positive amplification for *Yersinia enterocolitica* O:9 and two samples were positive for both. Table (5) also, demonstrates that three samples were positive for Yersinia out of 10 fecal samples while none were positive for Brucella. Moreover, 11 samples were positive for *Brucella* out of 18 stomach content samples and none were positive for *Yersenia*.

Table 1: Type & number of samples.

Animal status	No. of animal	Type & number of Samples				Total No. of samples
		Serum	Blood on (EDTA)	Stomach contents	Feaces	
Apparently healthy	7	7	7	-	-	14
Fevered	10	10	10	-	-	20
Diarrheic	10	10	10	-	10	30
Abortion	18	18	18	18	-	54
Total	45	45	45	18	10	118

Table 2: Primers used in Multiplex PCR reaction of *Brucella* and *Yersenia enterocolitica.O:9*.

Target	Name (strand)	Primer sequence (5 - 3)	Reference
Brucella	B4 F	5'- TGGCTCGGTTGCCAATATCAA- 3'	Baily *et al.,* (1992) Mukherjee *et al.,* (2007) Moussa *et al.,* (2011)
	B5 R	5'- CGCGCTTGCCTTTCAGGTCTG- 3'	
Yersinia	16S rRNA F	5'-AATACCGCATAACGTCTTCG-3'	Balakrishna *et al.,* (2012)
	16S rRNA R	5'-TCTGCGAGTAACGTCAATCC-3',	

Table 3: Cycling protocol of multiplex PCR for amplification of BCSP &16S rRNA genes of both *Brucella* & *Yersenia enterocolitica* respectively.

Target	Amplicon size	Cycling condition			No. of cycle
		Step	Temp.	Time	
		Initial denaturation	95˚C	5 min	One cycle
Brucella	223bp	Denaturation	94˚C	45 s	35 cycles
Yersinia	325bp	Anealing	55˚C	45 s	
		Extension	72˚C	1 min	
		Final extension	72˚C	10 min	One cycle

Table 4: Results of BAPA and RBPT on serum samples.

Test	BAPAT						RBPT					
	Positive scores				-Ve	Total	Positive scores				-Ve	Total
	1+	2+	3+	4+			1+	2+	3+	4+		
Number	8	8	14	1			3	12	7	1		
Total	31				14	45	23				22	45

BAPAT = Buffered Acidified Plate Antigen Test. **RBPT** = Rose Bengal Plate Test.

Table 5: Results of CFT and ELISA on serum samples.

Test	CFT								ELISA	
	Positive titer reciprocals						-Ve	Total		
	5	10	20	40	80	320			No. of +Ve	No. of -Ve
Number	5	3	6	7	1	2			26	19
Total	24						21	45	45	

Table 6: Results of Multiplex PCR.

Type of samples	No. of samples	No. of +ve Br	No. of +Ve Y.O:9	No. of Mixed
Blood on EDTA	45	19	5	2
Stomach content	18	11	0	0
F.S	10	0	3	0
Total	73	30	8	2

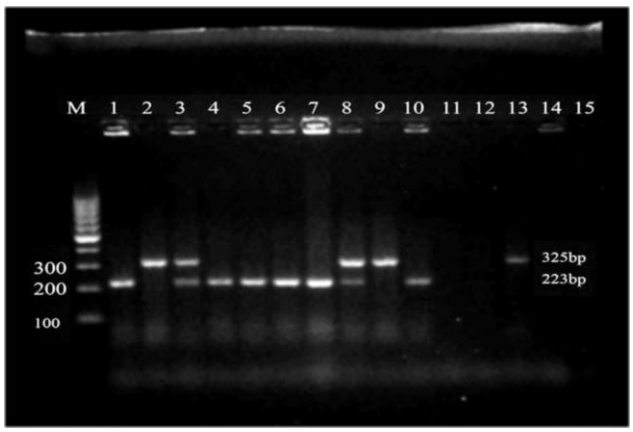

Fig 1: Shows ethidium bromide stained 1.5% agarose gel electrophoresis of Multiplex PCR products.
Lane M: 100bp DNA Ladder
Lane 1: Brucella positive control 223bp
Lane 2: Yersinia positive control 325bp
Lane 3: Mixed positive control
Lane 4- 14: Samples (lane 4-7 and lane 10 positive for Brucella , lane 8 positive for both , lane 9, 13 positive for Yersinia and lane 11,12, 14 negative samples)
Lane 15: Negative control

DISCUSSION

Accurate diagnosis of brucellosis in any species is fairly straightforward but may be very difficult in some cases. Because of the problems with isolation and identification of *Brucella* culture which relies upon a great deal of phenotypic traits: inefficiency, cost, danger and other factors, most laboratories prefer to use molecular biology as a diagnostic tool based on the amplification of genomic targets through different polymerase chain reaction (PCR) approaches (Fernando *et al.,* 2010). Moreover, these molecular approaches will soon be at the point of replacing actual bacterial isolation. It is rapid, safe and cost effective (Nielsen and Yu 2010).

Detection of Yersinia enterocolitica in clinical samples is still not sensitive and fast enough. Polymerase chain reaction (PCR) offers the advantages of sensitivity, specificity, and rapidity (Odinot *et al.*, 1997).

Inaccurate serological results causing incorrect diagnoses are a continuous problem when testing for infectious disease agents in animals or in human beings. Because of the genetic diversity of populations, some animals will respond with low antibody levels to exposure to *Brucella sp*., resulting in false negative results. Exposure to cross-reacting microorganisms may cause elevated antibody levels for various periods of time resulted in a false positive serological reaction, a major diagnostic problem in some areas where such microorganisms are endemic (Nielsen and Yu 2010).

Indirect enzyme-linked immunosorbent assays (ELISA) using S-LPS extracts or its O-chain have been extensively studied (Nielsen 2002) and may replace the Rose Bengal test (RBT) and CFT. These tests are the most sensitive for detecting cattle brucellosis, but they may yield false positive results with many other bacteria but only *Yersinia enterocolitica* O:9 is a significant cause of false-positive serological reactions (FPSR) in the diagnosis of bovine brucellosis (Gerbier *et al.*, 1997). Thus, *Y. enterocolitica* O:9 infections in cattle are troublesome and generate considerable additional costs in surveillance programs (Mun˜oz *et al.*, 2005).

Therefore, the aim of this work is to fulfill towards serological investigation and molecular differentiation between *Brucella* and *Yersinia enterocolitica* O:9 infections in cattle.

In our serological investigation the highest number of positive cattle was achieved by the presumptive BAPA. This test is supposed to exclude negative cases from further serological testing (Alton *et al.*, 1988) and positive cases should be confirmed. The RBPT behaved ineffectively in this study where it detected slightly less positive animals than did the confirmatory CFT. This could be attributed to the high specificity of the CFT that allows for a very low positive cutoff of 20 $IU.ml^{-1}$ and hence a sensitivity slightly superior than that of the RBPT whose cutoff value is 25 $IU.ml^{-1}$. Additionally, the CFT is more selective than the RBPT in terms of the detection of complement fixing IgG_1 (Ibrahim, 1982) which happens to be agglutinogenic at the acidic pH of the RBPT (Alton *et al.*, 1988) As expected from ELISA, its sensitivity was somewhere between the BAPA and the RBPT. This sensitivity is attributed to the fact that ELISA is a primary binding assay that detects mainly

all IgG isotypes regardless of their biological activity (Alton *et al.*, 1988).

Multiplex-PCR assays that performed in our work were standardized to amplify fragments of 223bp and 325bp corresponding to gene encoding a 31 kDa cell surface protein (BCSP) of *Brucella* and 16s rDNA gene as target for the detection of *Yersinia* species. The genus specific primer of the gene encoding a 31 kDa cell surface protein (BCSP) of *brucella* have been used with success to diagnose infection with brucella by various authors (Mukherjee *et al.,* 2007; Ayman and Nermeen 2010; Moussa *et al.,* 2011 and Jabbar *et al.,* 2012), none of whom ever related false positives. Also, a general-primer used to amplify a target sequence of 325-bp for *Yersinia* specific region of the 16S rRNA gene has been used to detect *Yersinia* spp., especially in blood samples (Sen and Asher 2001) and was recommended by (Arora *et al.,* 2012).

The result of multiplex PCR revealed that 19 blood samples gave positive amplification of 223bp for brucellosis and seven positive amplification of 325bp for *Yersinia enterocolitica* O:9 representing 26.92% of total positive *Brucella* of which two samples were positive for both *Yersinia* and *Brucella,* representing 7.69% this results agree with previous results of (Nagaraju *et al.,* 2001) and (Marcin *et al.,* 2012).

Also, multiplex PCR assay revealed the presence of three positive feacal samples for *Yersenia enterocolitica* O:9 in serologically positive Brucellosis this results confirm the previous results of (Reynaud *et al.,* 1993) who could isolate *Yersinia enterocolitica* O:9 from stool of cattle and goats had positive tests for brucellosis.

CONCLUSION

We can conclude that multiplex PCR assay for amplification of BCSP &16S rRNA genes of both *Brucella & Yersenia* can be successfully used for simultaneous detection and differentiation of *Brucella* and *Yersinia enterocolitica* O:9. *Y. enterocolitica* O:9 infections in cattle are seem to be a troublesome and generate considerable additional costs in Brucellosis surveillance programs. This study should be extended to a large scale population for proper identification of epidemiological status of cattle herd.

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التمييز السيرولوجى والجزيئي بين الإصابة بالبروسيللا واليرسينيا إنتيروكوليتيك O:9 في الأبقار

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يعتبر مرض الإجهاض المعدي (البروسيلا) من أهم الأمراض المشتركة التي لا تزال محل اهتمام كل من الأطباء البيطريين والبشريين في جميع أنحاء العالم وهى أيضا تمثل عائقا هاما في الاقتصاد القومي. في هذا البحث تم تجميع 118 عينة مختلفة (45 عينة سيرم ، 45 عينة دم على مانع للتجلط ، 18 عينة من محتويات المعدة للأجنة المجهضة وأيضا 10 عينات براز) من عدد 45 أبقار مصابة وأخرى غير مصابة ظاهريا من بعض الوحدات البيطرية بمحافظة الشرقية. تم فحص 45 عينة سيرم باستخدام طرق سيرولوجية مختلفة مثل (الروزبنجال الشريحى ، الأنتجين المخمد المحمض الشريحى ، اختبار تثبيت المكمل وأيضا اختبار الإليزا). باقي العينات (الدم ومحتويات المعدة والروث) فقد تم فحصهم باستخدام اختبار إنزيم البلمرة المتسلسل المتعدد. وقد كانت النتائج إيجابية للبروسيللا في 26 عينة من 45 عينة سيرم باستخدام اختبار الإليزا. بينما أظهرت النتائج 19 عينة (26,92%) إيجابية للبروسيلا من مجمل الحيوانات الإيجابية للبروسيللا بالاختبارات السيرولوجية و7 عينات إيجابية لليرسينيا من بينهم 2 عينة (7,69%) إيجابية لكل من البروسيللا واليرسينيا معا. من هذا نستخلص أن اختبار إنزيم البلمرة المتسلسل المتعدد يعد من الاختبارات الجيدة للكشف المتزامن الدقيق والسريع للبروسيلا واليرسينيا وأيضا التفرقة بينهما.