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POLYMERASE CHAIN REACTION (PCR) AS AN ACCURATE TECHNIQUE FOR DIAGNOSIS OF BRUCELLOSIS IN CATTLE

(With One Table and One Figure)

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اختبار أنزيم سلسلة عديد البلمرة كاختبار هام لتشخيص البروسيلا فى الأبقار

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اشـتملت هـذه الدراسة على إجراء اختبار التلبد الأنبوبى على مصل الدم لعدد ١٢٤ بقرة بمزرعة خاصة بمحافظة الفيوم و ذلك لمعرفة مدى إنتشار البر وسيلا. تم الكشف عن وجود المرض باستخدام هذا الاختبار فى عدد ٣٧ بقرة بنسبة ٢٩,٨%. كانت نسبة الإصابة عالية فـى الحيوانات البالغة عنها فى الحيوانات اليافعة. كانت الأعراض الإكلينيكية فى الحيوانات المصابة عبارة عن إجهاضات فى مراحل الحمل الأخيرة واحتباس فى المشيمة وإلتهابات فى الضـرع. أثبـت الفحص البكتريولوجى لعينات اللبن والأنسجة المختلفة والأنسجة المجهضة للحـيوانات الإيجابـية لاختـبار التلبد الأنبوبى عزل عدد ٩ عترات من ميكروب البروسيلا وبتصـنيف هذه العترات وجد أنها جميعا تحمل الخصائص البيوكيميائية للبروسيلا أبورتس. وبإجراء اختبار أنزيم سلسلة عديد البلمرة لعدد ٢ عينة دم من حيوانات سالبة لكل من اختبار التلـبد الأنـبوبى والعزل البكتريولوجى أعطى نتيجة إيجابية وقد أعطى هذا الاختبار نتيجة إيجابـية أيضـا عـند أجراءة على ٥ عينات لبن مأخوذة من حيوانات ايجابية لاختبار التلبد الأنبوبى وسلبية للعزل البكتريولوجى بالرغم أن هذا الاختبار قد أعطى نتيجة سلبية فى إنسان مخالط للحيوانات المصابة فى هذه المزرعة موجب لاختبار التلبد الأنبوبى. ومن هذه النتائج اثبـت اختـبار أنزيم سلسلة عديد البلمرة نتائج أدق وأكثر حساسية عن كل من اختبار التلبد الأنبوبى والعزل البكتريولوجى.

SUMMARY

At Fayoum Governorate, blood samples were collected from 124 Friesian cattle to determine the prevalence of brucellosis using Tube Agglutination Test (TAT). Among the examined animals positive titers (more than 1/20) were recorded in 37 animals with a prevalence rate of 29.8%. The prevalence of brucellosis was significantly higher ($p<0.05$) in adult (35.8%) than young (12.5%) animals. Infected animals showed

signs of late stage abortion beginning from the fifth months to the nine months, retained placentas and mastitis. Microbiological culture from the aborted fetuses, vaginal swabs and milk samples on tryptic soya agar after addition of brucella supplement revealed *Brucella abortus* in 9 animals out of the 37 serologically positive animals. Application of Polymerase Chain Reaction (PCR) on two blood samples obtained from serologically and bacteriologically negative cows showed positive PCR results. Moreover, PCR on 5 milk samples obtained from cows showed serologically positive and bacteriologically negative results gave positive PCR reactions. In addition blood sample was taken from human attendant in infected farm showed positive serological TAT (Titer 1/160) but results of PCR gave negative results. Finally it can be concluded that PCR is more sensitive technique than cultures and more specific than TAT.

Key words: *Cattle brucellosis, Diagnosis brucellosis*

INTRODUCTION

Animal and human health are inextricably linked. For millennia, humans have depended on animals for nutrition, socioeconomic development, and companionship (Acha and Szyfres, 1987). Brucellosis is one of the five most common bacterial zoonotic in the world and of economical losses. This disease is caused by the species of the genus *Brucella*, a homogeneous group of Gram-negative bacteria that are able to multiply within professional and non-professional phagocytes (Corbel and Brinley-Morgan, 1984; Riley and Robertson, 1984; Anderson and Cheville, 1986; Detilleux *et al.*, 1990a,b) Six species of genus *Brucella* are presently known: *B. abortus*, *B. suis*, *B. melitensis*, *B. ovis*, *B. neotomae*, and *B. canis* (Moreno *et al.*, 2002).

Brucellosis is usually transmitted to humans by ingestion of unpasteurized dairy products or by direct contact with infected animals (Altuglu *et al.*, 2002). Occupational disease is contracted by exposure of abattoir workers and veterinarians to infected animals especially aborted fetuses, fluids, membranes or urine (Bardenstein *et al.*, 2002 and Nimiri, 2003). Brucellosis is a disease of the sexually mature animals with predilection for placentas, fetal fluids and testes of male animals (Al-Majali, 2005).

Diagnosis via microbiological isolation is considered the "Gold *standard" against which other tests are compared (Morgan, 1977; Yagupsky, 1999 and Altuglu et al., 2002), however, it is* time consuming

and represent a hazard of human infection (McGiven *et al.*, 2003). Because of the use of antibiotics and the problems in culturing the accurate presumptive diagnosis usually depends on the indirect serological techniques in combination with clinical observations and case histories (Wright *et al.*, 1993 and Shapiro and Wong, 1999).

Serological techniques which based on the detection of antibodies to the lipopolysacharide (LPS) antigen is smooth *Brucella* strains are the main tool for brucellosis diagnosis and associated testing programmes (McGiven *et al.*, 2003). The main problem with brucellosis diagnosis has been the differentiation between vaccinal antibodies and those produced by field infection especially in situations where vaccination is a common practice and reliable records are not available from previous years (Dajera *et al.*, 1999).

Recently, several Polymerase Chain Reaction (PCR) assays to detect or differentiate *Brucella* strains have been reported (Baily *et al.*, 1992; Bricker and Halling, 1994; Leal-Klevezas *et al.*, 1995; Romero *et al.*, 1995 and Adone *et al.*, 2001). PCR, based on *Brucella*–specific primers, on blood samples is a potentially powerful tool (Fekete *et al.*, 1990). It showed higher sensitivity over serological test and blood culture and potential towards a rapid identification of *Brucella* strains in the blood and milk of infected animals (Leal-Klevezas, *et al.*, 2000).

Despite the preventive and control measures that exist in developed nations, there is still a high potential for transmission and spread of brucellae via animal products imported from developing nations (Acha and Szyfres, 1987). Thus, animal brucellosis poses a barrier for trade of animals and animal products and could seriously impair socioeconomic progress in the developing world (World Health Organization, 1986; Madkour, 1989 and Luzzi *et al.*, 1993). So, the aim of the present work was directed to determine the prevalence of brucellosis in cattle and the role of PCR as recent technique to overcome the drawbacks of the other conventional diagnostic techniques which are commonly used in brucellosis diagnosis.

MATERIALS and METHODS

Animals:

One hundred and twenty four Friesian cattle belonging to private farm at Fayoum Governorate were used in this study. Only cattle >6 months old were tested. These animals were not subjected to any vaccination against brucellosis.

Blood samples:

Individual blood sample was collected from each animal without anticoagulant in a vacutainer tube. The sera were separated by centrifugation at 3000 r.p.m. for 15 minutes and stored at –20° C until testing. Moreover, two heparinized blood samples obtained from serologically and bacteriologically negative cows were collected for PCR. In addition one heparinized blood sample was taken from human attendant in the same farm showed positive tube agglutination test (Titer 1/160) to be tested by PCR.

DNA extraction (blood):

Blood samples DNA was extracted as described by Sambrook *et al.* (1989) using phenol-chloroform-isoamyl alcohol and precipitated by absolute ethanol, washed with 70% ethanol and dried under a vacuum. DNA pellet was resuspended in 25 µl of sterile distilled water. Digestion of RNA was done by using Rnase enzyme at 37° C for 1hour. One microgram (µg) of this DNA suspension was added to the PCR mixture.

Milk collection technique:

Five milk samples were collected from 5 cows gave positive results on serological test and negative results on culture to be used for PCR according to the method described by Sreevatsan *et al.* (2000). Briefly, All milk samples were obtained from animals during their routine milking time. This approach prevented disruption of farmers' day-to-day on-farm operations and simplified sample collection. The samples from each animal were obtained from all four quarters of the mammary gland. Collections were done after thorough disinfection of the teat area with povidone iodine. Two to three strippings of milk from each teat were drawn into a sterile test tube, and the tubes were capped and stored on ice until further use.

DNA extraction (milk):

DNA was extracted according to the method described by Vitale *et al.* (1998).

Serological examination:

All collected serum samples were tested for presence of *Brucella* antibodies using Tube Agglutination Test (TAT) by using sterile phenol saline solution according to the method described previously by Alton *et al.* (1988) using *Brucella abortus* antigen obtained kindly from Serum Research and Vaccine Institute

Bacteriological examination:

Hundred microliters of stomach contents and swabs of lungs, livers and spleens from aborted fetuses and swabs from the vagina of aborted cows were subjected to direct microscopy and culture on tryptic soy agar duplicate plates supplemented with Brucella supplement (Abassia), one incubated in 10% CO_2 incubator at 37°C for 1 week and the second in normal atmosphere. Plates without growth must be left till 30 days before discarded as negative. Suspected colonies were subjected to further identification by morphological characters of the resultant colonies, microscopical examination of smears done from suspected colonies stained by Gram stain and Modified Ziehl nelson stains and by reaction with *brucella* positive and negative sera (Alton *et al.*, 1988).

Biochemical identification:

The resultant *Brucella* strains were examined for H_2S production using a stripe of dried lead acetate paper, their CO_2 requirements, and their ability to grow in the presence of thionin at concentration 1/25000, 1/50000 and 1/100000 and carbol fuchsin at concentration 1/5000 and 1/100000. Moreover, oxidase test using oxidase reagent, urease test using Christein 's urea agar and catalase test using 3% H_2O_2 were done according to Alton *et al.* (1988).

Amplification and detection of *Brucella* DNA by PCR:

Two oligonucleotides primers used for amplification of *Brucella* DNA prepared according to the sequences of highly conserved region that coding for outer membrane protein (OMP) (Baily *et al.*, 1992). PCR products are 216 base pairs. Primers sequences were primer 1 (P1 6633 TCGGTTGCCAATATCAA 793-809), and primer 2 (P2 6634 CTTGCCTTTCAGGTCTG 1008-992). The primers synthesized by MWG-Biotech AG, Holle & Huttner GmbH, Germany. Reaction mixture of 50 µl volume containing 10x PCR buffer (500 mM KCl; 100 mM Tris HCl pH 9.0; 1% Triton x –100; 1.5 mM $MgCl_2$ Promega, USA), 20 mM dNTPs (Boehringer Mannheim, Germany), 100 pmole of each primer 2.5 unit of *Taq* DNA polymerase (Promega, USA) and 1 ug of extracted DNA. The positive control contained 100 ng of *B. abortus strain 2308* DNA as a template, and the negative control consisted of sterile water instead of the DNA template. The thermal cycler (MJ research, USA) was programmed as first initial denaturation at 94° C for one min followed by 39 cycles consisting of 94° C for one minute, 55° C for one minute and 72° C for one minute and 10 minutes at 74° C for final extension. The amplified product was resolved using 1.5 % agarose gel electrophoresis that stained with ethidium bromide and photographed

by photo-documentation system (UVP, USA) and analyzed by Gel-pro 3.1 Analyzer (MEDIA, USA).

Statistical analysis:

Chi-square was done to the obtained data according to the method described by Snedecor and Cochran (1980). Probabilities less than 0.05 were considered significant.

RESULTS and DISCUSSION

Brucellosis is a transmissible disease that is considered to be of socio-economic and public health importance and can be a barrier to trade of animals and animal products (Office International Des Epizooties, 2000)

Among the examined 124 animals using TAT, positive titers (1/20 or more) were recorded in 37 animals with a prevalence rate of 29.8% (Table 1). Different prevalence rates were previously recorded as 50% by Fahmy and Bendary (1970); 8.2% by Hamdy (1992); 8.5% by El- Baumey (1993); 30.8% by El Sheery (1993); 9.3% by Abdel-Gawaad (1996); 21.72% by Abou-Zaid and Mehanna (1998); 21% by Montasser (2001); 11% by El-Gamel (2004); 24.27% by Hegazy (2004) and 55.6% by Bernarda *et al.* (2005). This variation in the disease prevalence may be attributed to the animal population; their susceptibility, vaccination status and the hygienic measures applied in each localities.

The prevalence of brucellosis was significantly higher ($p<0.05$) in adult (35.8%) than young (12.5%) animals. Similar results were reported previously by Ammar (1990); Ammar (1995) and Kubuafor *et al.* (2000). This may be attributed to the fact that, *brucella* organisms need for their multiplication and propagation a mature sexual organs (Arthur, 1975 and Al-Majali, 2005).

Table 1: Prevalence of brucellosis among the examined animals.

Age	No. examined animals	No. serologically positive animals	%
Under 2 years	32	4	12.5
Over 2 years	92	33*	35.8
Total	124	37	29.8%

* Reactors at a titer of 1/20 or above.

Brucella infection in cattle is characterized by an inflammatory response affecting cells of the reticulo-endothelial system and of the placenta during pregnancy. This often results in the death and expulsion of the fetus between the fifth and eighth month of gestation. During abortion, large numbers of brucellae are released which may, in turn, cause the infection of other animals in the herd (Manthei and Carter, 1950). Infected animals showed some reproductive abnormalities in the form of late stage abortion beginning from the fifth months to the nine months, retained placentas and some cases of mastitis. Although some serologically positive animals were not showed apparent signs. Similar signs were reported previously by Enright (1990); Madkour (2001) and Hegazy (2004).

Direct microscopy on the specimens obtained from the aborted fetuses, abomasums contents, vaginal swabs and milk samples failed to demonstrate the microorganisms. On the other hand, microbiological culture on tryptic soy agar after addition of brucella supplement revealed 9 isolates (5 from fetal abomasums and aborted fluids, 3 from milk samples and 1 from lung specimens) out of the 37 serologically positive animals. All *brucella* strains were identified as *Brucella abortus* by biochemical characteristics such as positive oxidase, urease, catalase, H_2S production, in addition to positive Carbon dioxide requirement and growth in the presence of thionin and basic fuchsin at the different concentrations. This come in agreement with the results of El-Gibaly (1969); Sayour et al. (1970); Abdel-Galil *et al.* (1986); Hosein (1987); Refai *et al.* (1990); Salem and Hosein (1990); Hamdy (1992) and Montasser (2001) who isolated different biotypes of *B. abortus* in Egypt.

From the above mentioned data, it can be concluded that, Although the definitive diagnosis of infectious disease can be accomplish only through the direct demonstration and identification of the causative agent(s) by culture and isolation techniques, sometimes this may be difficult and beyond the expertise and capabilities of diagnostic laboratories. Also microbiological procedures are time-consuming, cumbersome and carry a great risk of infection for laboratory technicians particularly those in developing countries (Wright *et al.*, 1993; Adone *et al.*, 2001; McGiven *et al.*, 2003 and Probert *et al.*, 2004). In addition to, Prolonged incubation period, special growth media, and subcultures are required for the isolation of these fastidious, slow growing bacteria (Shapiro and Wong, 1999). However, cultures are not always positive when other tests are positive (Romero *et al.*, 1995).

The standard tube agglutination test (STAT) which used routinely in serological diagnosis, might be gave false negative results due to blocking antibodies in addition to its fall to detect early or latent infections (Marin *et al.*, 1989; Leal-Klevezas *et al.*, 2000 and Ardic *et al.*, 2005) or false positive results due to cross-reacting with other common antigenically related bacteria (Leal-Klevezas *et al.*, 2000). In this study, application of PCR on two blood samples obtained from serologically and bacteriologically negative cows gave positive PCR results. Similar results were obtained previously by Morata *et al.* (2001) who obtained PCR positive results from serologically negative patients. Moreover, PCR on 5 milk samples obtained from cows showed serologically positive and bacteriologically negative results gave positive PCR reactions. In addition blood sample was taken from human attendant in infected farm showed positive serological TAT but results of PCR gave negative results (Figure 1). This might be attributed to the cross-reactivity between the smooth *Brucella* spp. lipopolysaccharide and various organisms of other genera (Corbel, 1985). Amongst these, *Yersinia enterocolitica* O:9 shows the most complete similarity to the O-chain of *Brucella* (Caroff *et al.*, 1984 and Nielsen *et al.*, 2004). *Y. enterocolitica* O:9 has been identified as a fairly common cause of cross-reactions in pigs and human (Ahvonen and Sievers, 1969 and Akkermans and Hill, 1972), and was described as able to induce comparable serological responses in ruminants (Corbel, 1985; Garin-Bastuji, 1993; Reynaud *et al.*, 1993; Hilbink *et al.*, 1995; Arthur, 1997; Pouillot *et al.*, 1998 and Garin-Bastuji *et al.* (1999). Also similar findings were obtained by Weynants *et al.* (1995 and 1996); Gerbier *et al.* (1997) and Godfroid *et al.* (2002) who recorded low specificity of the serological tests when used to analyze sera from *Yersinia enterocolitica* O:9 infected heifers.

Finally it can be concluded that diagnosis of brucellosis must be depends mainly on an accurate technique such as PCR where, cultures are not always positive when other tests are positive. In addition to the fact that PCR assay is more sensitive than conventional cultures. Also the most commonly used serological test (TAT) may give false negative and positive reactions.

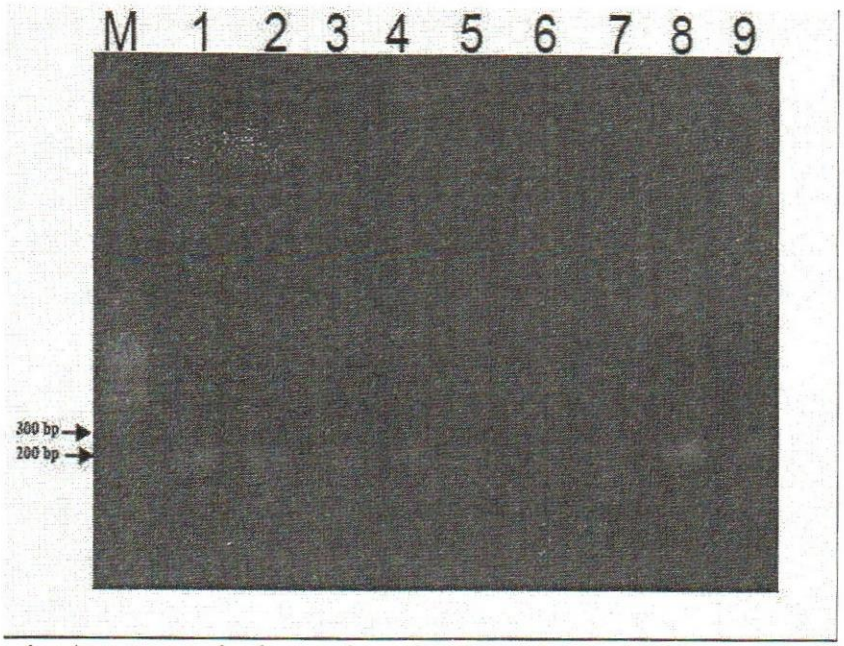

Fig. 1: Agarose gel electrophoresis. Lane M is 100-base pair DNA ladder size marker; lane no. 1 is a control positive; lane no. 2, 3, 4, 7 and 8 are five milk samples; lane no. 6 is a control negative, lane no. 5 and 9 are blood samples. Note that all samples are PCR positive with a target size of 216 bp.

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