Journal of Applied Sciences

ISSN 1812-5654

science alert

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OPEN ACCESS Journal of Applied Sciences

ISSN 1812-5654
DOI: 10.3923/jas.2019.77.81

Research Article
Diagnosis of *Brucellosis* in Recently Aborted Ewes Using Serological Tests and Polymerase Chain Reaction

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Abstract

Background and Objective: Generally, abortion cause enormous economic losses in livestock animals. The most important pathogens involved in the abortion of ewes are *Brucella melitensis*, *Campylobacter fetus*, *Salmonella abortus ovis* and *Chlamidophila abortus*. In Assiut governorate, five flocks of mixed breed ewes showed unexplained high percentage of abortions (33.3%) and the etiology of abortions wasn't well understood. Therefore, the objective of the current study was to estimate the cause of late abortion in that flocks in Assiut governorate (Upper Egypt). **Materials and Methods:** A total number of 94 recently aborted ewes and 47 aborted fetuses with related placenta were examined and we correlated its possible association with *Brucella melitensis*, which is the most important abortive diseases in sheep. Serum samples were tested by Rose Bengal and ELISA for brucellosis. The infected tissues and serum were also used in polymerase chain reactions (PCR) for detection of DNA of *Brucella* spp. **Results:** The results revealed that serological tests were positive in (21.28%) of examined cases, while *Brucella* spp. DNA was detected in 34.04% of serum samples and in 25.5% of tissue samples. **Conclusion:** It concluded that there was an association between *Brucella* infection and abortion in sheep in Assiut governorate, PCR could be accurate method for diagnosis of Brucellosis, thereby could control the infectious diseases in sheep and minimize reproductive losses.

Key words: Abortion, *Brucella* spp., PCR, serological test, sheep

Citation: Hassan Ali Hussein, Ragab Hassan Mohamed, Aml Mohammed Abdel-Ra'ouf, Eman Mahmoud Abu-Elnaga, Rasha Salah Mohamed, Asmaa Abd El-Nasser Hussein and Axel Wehrend, 2019. Diagnosis of *Brucellosis* in recently aborted ewes using serological tests and polymerase chain reaction. J. Applied Sci., 19: 77-81.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Although a gradual increase in the abortion rate in ovine flocks may be noted over a period of many years, a sudden and dramatic increase in it is more commonly seen especially when infectious agents are the cause of abortion. Brucellosis continues to be a great challenge to the development of dairy production in developing countries. It is reported that a most common epidemic infection in Mediterranean and middle eastern countries, Asia, Latin America and south Europe is Ovine and caprine brucellosis[1]. Cross transmission from *Brucella* can occur between different species like cattle, sheep, goat, camel and others and it affects almost all domestic species[2]. In Egypt, it is still endemic and one of the most economically devastating diseases for animal and human. As it causes a great loss among offspring, decreased productivity in addition to causes serious human health problems[3] and diminished levels of milk production[4,5]. *B. melitensis* isolated from sheep and goats and it is indicated from studies done in various parts of Egypt[6,7] and cattle[8]. Sheep and goats are considered the classical hosts for *B. melitensis.* Abortion in sheep, caused mainly by *Brucella melitensis* or rarely by *B. ovis*[9,10], *B. ovis* does not have zoonotic potential whereas *B. melitensis* does[11]. *Brucella* can survive up to 15-25 days on a pasture, transmission to the ewe can happened during contact with infected rams or infected material with *Brucella* through mucous membrane (vaginal, preputial and conjunctival)[12]. Soil contaminated with abortion secretions may threat animal health and human[13-15]. Ewe can abort in the third trimester, have stillbirth or give birth to a weak lamb although infection in ewes are generally asymptomatic. Ewes clear the bacteria within a few weeks following an abortion. The serological methods are usually employed for diagnosis of Brucellosis, however the serological response in sheep are not conclusive, can be unspecific because not all infected animals produce detectable levels of antibodies and because cross-reactions with antigens other than those from *Brucella* can give false-results[16]. Thus, diagnosis of *Brucella* should be confirmed by bacterial culture or PCR[17,18]. Culture methods are not always successful and handling of micro-organism is hazardous also they are time-consuming[19], otherwise isolation rate is very low even in experienced laboratories[20]. There are limited published reports of brucellosis in ewes in Assiut. Therefore, the objective of this study was to investigate if there was relation between *Brucella* spp. infection among the sheep flocks showed high percentage of late abortion in that governorate by detection of *Brucella melitensis* DNA with molecular techniques.

MATERIALS AND METHODS

Animals and management: Five hundred and forty mixed breed ewes in Assiut governorate (Upper Egypt) at March and April in the years of 2015 and 2016 showed unexplained high percentage of abortions (180 ewes, 33.3%) investigated in this study. From them 97 freshly aborted ewes were included. The ewes aged between 1.5-5 years with mixed parity (include primipara and pluripara). Clinical examination of aborted ewes showed no clear clinical symptoms as fever or decrease in food intake. Rams were together with ewes; this means all pregnancies were resulted from natural mating. The investigation carried out at villages in Assiut governorates (22°42' latitude and 30°45' E longitude). The owner's complain that ewes on those farms exhibited a persistent abortion problem.

Serum samples: A total 94 blood samples collected from very recently aborted animals that not previously vaccinated against *Brucella*. Blood samples obtained aseptically from the jugular vein then centrifuged for 10 min at 1500 rpm. Serum collected and stored at -80°C until further use.

Tissue samples: All samples taken approximately 1-3 days after abortion, a total number of 47 aborted fetuses and its related placentas admitted to the Department of Theriogenology, Faculty of Veterinary Medicine at the University of Assiut. Necropsy conducted on the aborted fetuses. Livers of six aborted fetuses showed diffuse necrotic foci. Tissue samples taken from the several fetal organs including placenta, liver, lung, kidney, spleen, heart, stomach fluid and stored at -80°C until DNA extraction. The corresponding number of blood samples analyzed.

Serological test: The samples tested using rose bengal plate test (RBPT)[21]. The sera and antigen-Rose Bengal (RB) obtained from Atlas Medical (www.atlas-site.co.uk.). The kits brought to room temperature before testing using antigen micropipette a drop (30 µL) of the serum and placed on dry white enamel plat, one drop of RB antigen (30 µL) was added to one drop of serum where thoroughly mixed in a circular movement using tooth pick or glass red. The plate shacked by hand for 4 min and any agglutination that appeared within this time recorded as a positive reaction.

Enzyme-linked immunosorbent assay (ELISA): The assay was done using competitive ELISA Kits "COMPELISA 160 and 400°C. ELISA Kits" From APHA Scientific;

aphoscientific@alpha.gsi.gov.uk used for detection of antibodies against *Brucella* spp., in serum samples. the procedures followed as manufacture instruction.

DNA extraction: DNA extraction from frozen tissue samples (fetal tissues, placentas and serum) were performed using QIAamp® DNA Mini and Blood Mini Kit and according to the manufacturer's procedure (Qiagen, Cat. No. 51304).

Polymerase chain reactions (PCR): DNA samples tested for detection of *Brucella* spp. by PCR reactions performed using 13 µL of PCR mix (Promega, USA), 0.75 µL of a 25 µM solution of each primer (Table 1) and 1 µL of DNA template. Thermal profile used was initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 1 min. *Brucella* annealing at 55°C for 30 sec. PCR products resolved by electrophoresis in a 1% agarose gel stained with ethidium bromide. Positive, negative controls and 500 bp DNA Marker were included in all reactions.

RESULTS

Comparison of PCR with RBT and ELISA: A total number of 32 (34.04%) *Brucella* positive samples were detected only in PCR test of the 94 serum samples. PCR products with a molecular size of 450 bp Indicative of *B. melitensis* DNA were obtained of the 94 serum samples, 32 (34.04%) and 12 (25.5%) of fetal tissue tested positive by PCR. When tested and PCR results were compared to Serological tests like ELISA and RBT 20 samples was positive (21.28%) from 94 serum samples tested (Table 2, Fig. 1).

DISCUSSION

In present study, higher prevalence of brucellosis in ewes was noticed by PCR (34.04%) in serum samples and (25.5%) in fetal tissue, followed by ELISA (21.28), RBPT (21.28%). *Brucella* infection has been considered as a major problem of wild and domestic animals. It is recognized that Brucellosis is a major cause of worldwide zoonotic disease that is recognized as a major cause of heavy economic losses to the livestock industry and poses serious human health hazard[3]. The *B. melitensis* is the main aetiologic agent of brucellosis in small ruminants. Ewes' and nanny-goats' aborted foetuses and products

Table 1: Primer sets for conventional PCR for *Brucella melitensis*

Primers	Primer sequences (5'-3')	Amplicon size (bp)
BMEI0535 f	GCG-CAT-TCT-TCG-GTT-ATG-AA	450
BMEI0535 r	CGC-AGG-CGA-AAA-CAG-CTA-TAA	

Table 2: Positive result of serological and molecular tests

	RBPT			ELISA			PCR		
Samples	No.	Positive	Percentage	No.	Positive	Percentage	No.	Positive	Percentage
Serum	94	20	21.28	94	20	21.28	94	32	34.04
Fetal tissues	-	-	-	-	-	-	47	12	25.50

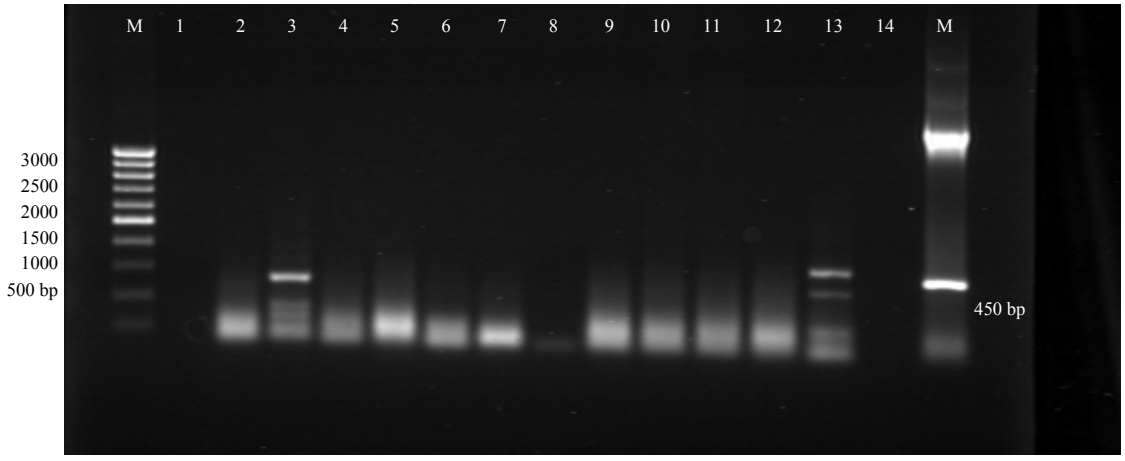

Fig. 1: Agarose gel electrophoresis of PCR product from *Brucella meltensis* product. M: Molecular size marker DNA ladder (500, 1000, 1500, 2000, 2500 and 3000 bp), Lane 1: Negative control, Lane 2: Positive control, Lanes 3-7 and 9-13: Positive products of *Brucella meltensis*, Lanes 8 and 14: Negative products of *Brucella meltensis*

derived from sheep and goats remain the main source of infections. Ovine and caprine brucellosis reported as a most common epidemic infection in Mediterranean and middle eastern countries, Asia, Latin America and South Europe[1,2]. Serological tests done on serum used for screening of brucellosis and play an important role in surveillance programs of the disease[21]. In Egypt, Rose Bengal plate test and ELISA used to determine the prevalence of brucellosis in different animal species as recorded by different studies[22,23]. In our study, clinical presentation of abortion and strong seropositive results 20/94 (21.28%) led to the diagnosis of brucellosis. serological diagnosis from freshly aborted animals may fail because low antibody titers against *Brucella* infection[24], or absences of antibody in some animals serum[13]. Moreover, laboratory confirmation of Brucella infection requires isolation of bacteria or detection of *Brucella* DNA by PCR[25]. Thus, the accurate diagnostic window of Brucella should be complemented by bacteriological or molecular diagnosis[18,26]. *Brucella* organisms were not isolated in this study as Brucella culturing is hazardous and the technique is restricted to few laboratories in Egypt[13]. Detection of Brucella DNA by PCR test from serum samples of corresponding ewes recorded in 32/94 cases (34.04%) and in tissue samples was 12/47 (25.5%). The advantages of PCR technique are fast, safe and unaffected by contamination by other microbes that might be present in the tissue samples used for isolation, that explain the superiority of the PCR assay as a diagnostic methods of brucellosis in serum and tissues of infected sheep and failure of serological test to recognized and detect brucella recently in aborted ewes. This study discovered the using of PCR that can be beneficial for detection of *Brucella* DNA in seronegative animals and this study will help the researchers to uncover the critical areas of early *Brucella* detection that many researchers were not able to explore. Thus, a new theory on may be arrived at.

CONCLUSION

This study concluded that PCR able to detect *Brucella* DNA in seronegative animals and it proposed to use PCR even as a tool for routine diagnosis especially in recently aborted ewes. This indicated that the sensitivity of the PCR assay was higher than that of the serological methods.

SIGNIFICANCE STATEMENT

This study discovered the using of PCR that can be beneficial for detection of *Brucella* DNA in seronegative animals and this study will help the researchers to uncover the critical areas of early *Brucella* detection that many researchers were not able to explore. Thus a new theory on may be arrived at.

ACKNOWLEDGMENT

The authors thank all workers in the molecular biology research unit of Assiut University, for their help with this work.

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