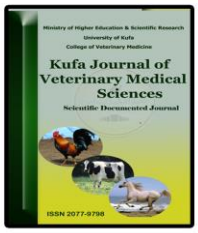
Kufa Journal for Veterinary Medical Sciences Vol.(5). No.(2) 2014

Kufa Journal for Veterinary Medical Sciences

www.vet.kufauniv.com



Molecular Diagnosis of *Coccidioides Posadasii* from Aborted Placenta of Ewes by RT-PCR

Bushra Hamza Fares* Ban A. Abdulmajeed**

Hameed Abdulhussein Mejbel***

Collage of vet. Medicine /kufa University
Collage ofMedicine /Nahrain University
Najaf.vet. hospital

Abstract

Diagnosis of diseases depends on histology using formaldehyde-fixed paraffin embedded tissue .The polymerase chain reaction (PCR)helps the pathologists to confirm the diagnosis of many disease including mycotic infections.
Aborted placentas from 40 ewes were formalin fixed, paraffin embedded and sectioned. Tissue sections were stained with haematoxylin and eosine, and periodic acid Schiff (PAS) for histopathology examination. Fresh tissue parts were cultivated ,subjected to DNA extraction for the purpose of real-time PCR amplification.
Histopathological examination of placental tissue revealed extensive necrotizing placentitis with the presence of spherule of *Coccidioides Posadasii*in the necrotic area. Only eight samples gave positive growth for *coccidioides Posadasii* out of forty aborted placental samples.
DNA amplification by real-time PCR revealed positive amplification for the eight samples which confirmed the detection of *coccidiodes posadasii***.**

Key words: *Coccidioides posadasii*, real-time PCR, necrotic placentitis, ewes.

التشخيص الجزيئي لفطر *Coccidioides Posadasii* من المشائم المجهضة للنعاج باستخدام تقنية RT-PCR

بشرى حمزة فارس بان عباس عبد المجيد حميد عبدالحسين مجبل

كلية الطب البيطري \جامعة الكوفة
كلية الطب \جامعة النهرين
المستشفى البيطري في النجف الاشرف

الخلاصة:

يعد الاعتماد على علم الانسجه احد أهم طرق تشخيص الأمراض وذلك باستعمال طريقة النسيج المثبت بالفورمالين والمغمس بالبرافين .كما ويساعد تفاعل البلمره المتسلسل في تأكيد تشخيص عدة أمراض منها الإصابات الفطرية.

تم دراسة 40 نموذج لمشيمة نعاج مجهضة ,حيث تم اخذ نموذجين من كل مشيمة احدهما للتقطيع النسيجي ,حيث تم استعمال صبغة haematoxylin and eosine وصبغة periodic acid Schiff للفحص النسيجي., والأخر للزرع الفطري واستخلاص الحامض النووي لغرض أجراء فحص RT-PCR .

اظهر الفحص النسيجي المجهري لأنسجة المشائم المجهضة وجود بقع واسعة متموته والتهاب مع ملاحظة وجود spherule of *coccidioides Posadasii* في المناطق المتموتة في ثمانية نماذج من النماذج الأربعين المدروسة, وتم تأكيد تشخيص فطر *coccidioides Posadasii* للنماذج الثمانية من خلال فحص RT-PCR

Introduction:

One of the major causes of abortion in animals is mycotic placentitis. Worldwide, it tends to occur sporadically. Occasionally, it may affect a significant percentage, about 10-20% of pregnant animals in a herd. Mycotic placentitis usually occurs in the third trimester of pregnancy.(1). An immunodeficient state of the host may facilitate hematogenous spread. Corticosteroid therapy ,prolonged antibiotic treatment,local treatment of vagina and cervix, infection with viruses such as Bovine Viral Disease and Infectious Bovine Rhinotrachitis can be regarded as immunosuppressive conditions that contribute to this spread,(2) as well as metabolic disturbance and stress.(3) In order to diagnose mycotic abortion, samples from the abortus are submitted to microscopical examination and cultivation. These are time consuming and lack sensitivity.(4) On the other hand, serological test can also be used but these have variable sensitivity and specificity(5,6). Histopathological examination is essential to confirm the mycotic infection from the morphological details of fungi. However, the appearance of hyphae in tissue sections is altered by a number of factors rendering it difficult for some investigators to determine the exact causative agent. (7)

The present study was carried out to detect C*occidioides posadasii* in aborted placenta by RT-PCR.

Materials And Methods

This is a cross sectional study whereby 40 ewes which sustained abortion were collected from various parts of Al-Najaf city**.** The aborted placentas were divided into 2 parts. One half was kept fresh to be sent for routine diagnostic procedures of direct examination and

cultivation in the laboratories of veterinary medicine\baghdad university. The second half was immersed into 10% formalin. Formalin fixed paraffin embedded tissue blocks were submitted for sectioning. Two slides were made from each block. One of them was stained with hematoxyline and eosine and the other with periodic acid Schiff stain for the purpose of histopathological examination. The fresh parts were cultured on Sabouraud dextrose agar in slant tubes at 30°C for 5 days. The cultures were identified by colony morphology and microscopy of typical arthroconidia. Sterile water was added to the cultures; hyphae and arthroconidia were taken with a sterile wooden stick from colonies and dissolved in water. After inactivation by boiling for 15-20 minutes, two samples of 200 _µl of the latter suspension were used for DNA extraction. Genomic DNA was extracted according to(Zimmermann *et al*., 1994). Using a UV spectrophotometer, the optical density at 260 nm and 280 nm was measured for each DNA sample. DNA concentration was measured applying the equation:

DNA concentration= 50 x O.D. reading at 260 nm x dilution factor.

The purity was determined by the ratio of O.D. at 260 nm/ O.D. at 280 nm. Amplification of DNA samples was carried out with specific

Primers for *coccidioides posadasii*.

Primers had the following sequences:

5_--GCTGAAGCTCTAGCTGCTCT -3_ (forward) and

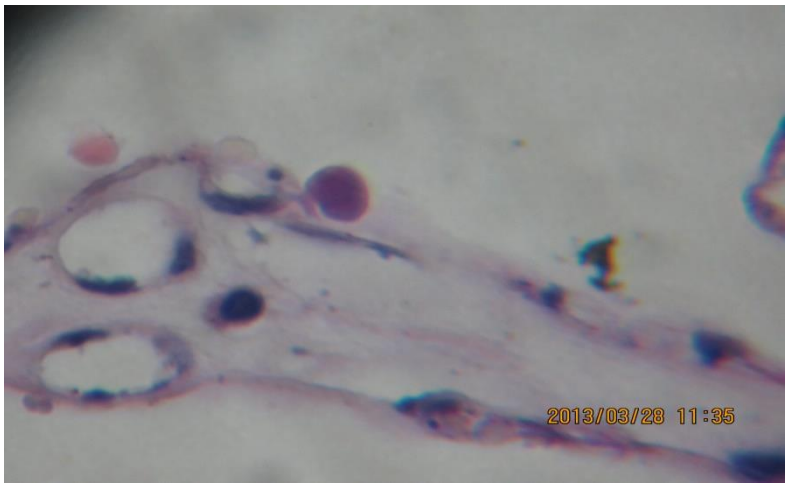
5_-GACAACCGGGGTTGGAGAAT3_ (reverse).

Real-time detection was performed on the qPCR Agilent technology 3000. Cycling parameters consisted of 10 µl KAPA SYBER* FASTqPCR Master Mix(2X), 0.4 µl forward primer(10uM),0.4 µl Reverse primer(10 µM), 0.4 d UTP (10µM), 0.4 ROX High / low ,and 3 µl template DNA made up to 20 ul with de-ionized water. The SYBER program was selected and cycling conditions were as follows:10 min at 95ºC,40 cycles of 30 seconds at 95ºC,1 min at 55ºC and 1 min at 72º C followed by a melt curve starting at 65º C rising to 94ºC at 0.3 per second. Data analysis included Ct values recording and plotting amplification curves for all samples.

Result:

Histological examination of the clinical isolates demonstrated the presence of spherules of *Coccidioides spp.* in 8 out of 40 samples, fig-1,2.

All of the 40 cultivated specimens revealed fungal growth. Only 8 samples gave positive growth of *coccidioides* spp. on cultivation as was proved from direct examination of smears made from the growths.



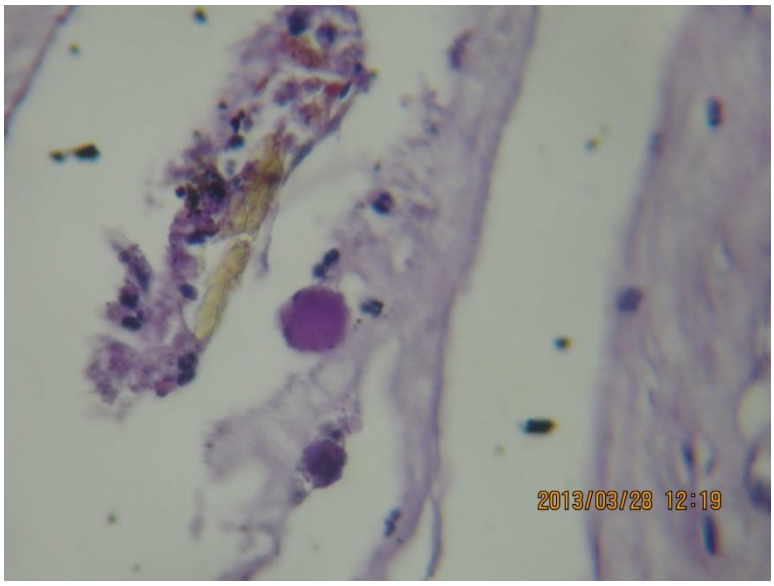
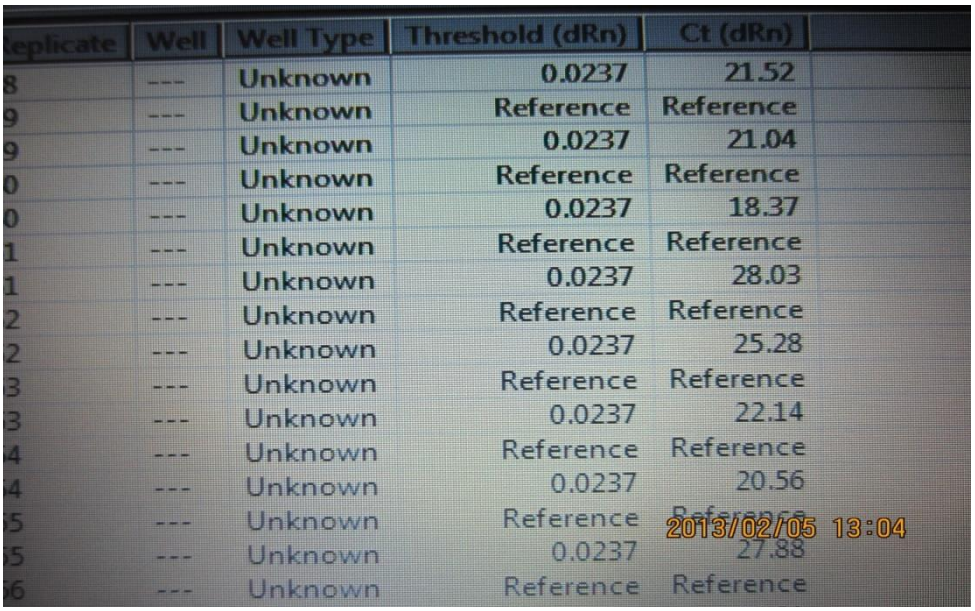


Fig:1,2. Histopathological section in the placenta of aborted sheep shows a spherule of C*occidioides spp*. in the necrotic debris (arrow). The section was stained by PAS stain (X40).

Extracted DNA from the isolats had a range of concentration from(0.116) ng/ml to(o.272)) ng/ml at 260 nm by using Nano Drop system.

DNA amplification by real-time PCR revealed positive amplification in the 8 samples suggesting the isolates to be of *C. posadasii.* The cycle threshold (ct) values for the amplified samples ranged from 21.52 - 27.88.



eplicate	Well	Well Type	Threshold (dRn)	Ct (dRn)
8	---	Unknown	0.0237	21.52
9	---	Unknown	Reference	Reference
9	---	Unknown	0.0237	21.04
0	---	Unknown	Reference	Reference
0	---	Unknown	0.0237	18.37
1	---	Unknown	Reference	Reference
1	---	Unknown	0.0237	28.03
2	---	Unknown	Reference	Reference
2	---	Unknown	0.0237	25.28
3	---	Unknown	Reference	Reference
3	---	Unknown	0.0237	22.14
4	---	Unknown	Reference	Reference
4	---	Unknown	0.0237	20.56
5	---	Unknown	Reference	Reference
5	---	Unknown	0.0237	27.88
6	---	Unknown	Reference	Reference

Fig. 3(A). Amplification of DNA samples with specific primers of *Coccidioides posadasii*.

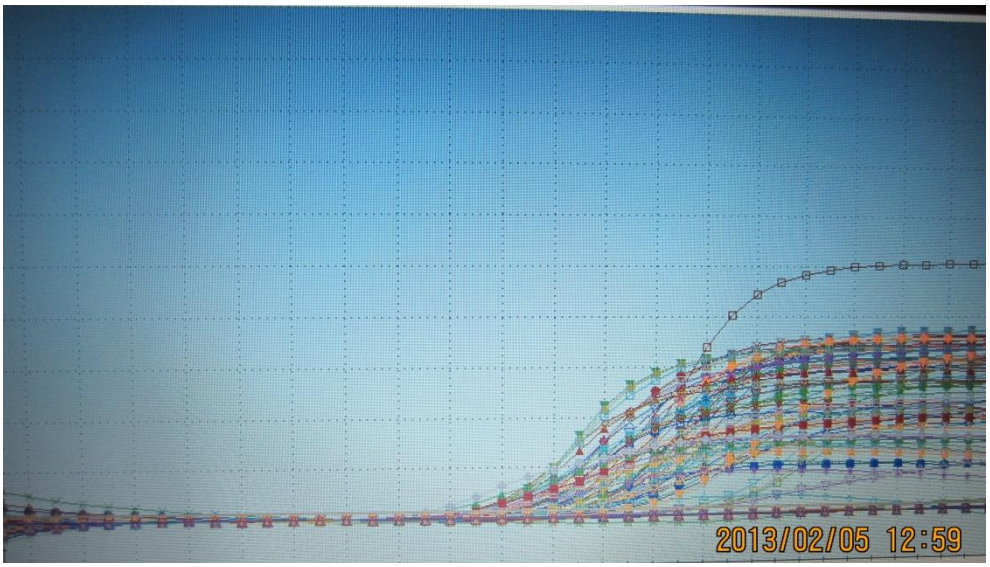


Fig:3(B)Post amplification melting curve analysis of *Coccidioides Posadasi* by RT-PCR

Discussion

Fungi infection in the genital tract of an animal can produce reproductive failure either by direct infection and necrosis, or by producing toxic metabolites (mycotoxins)which are consequently ingested and absorbed. Mycotic abortion is commonly caused by both *A. fumigatus* (62%) and *C.albicans*(2%) that normally present in the cervico vaginal cavity of dairy cows with or without reproductive disease(8).

To overcome the problem of animal loss due to this type of infection, many diagnostic methods have been introduced which did not prove to be sufficiently sensitive and specific to enable an early and effective diagnosis of the mycotic diseases.The need for an optimal and specific diagnostic method has led to the application of the polymerase chain reaction for this reason. (9) Fungi are ubiquitously present in the environment so that they might be contaminants in clinical specimens. In order to confirm their participation in abortion, they should be found in histopathological examination of the placenta (necrotizing placentitis) or fetus in addition to the routine microbiological diagnosis. (1) In the present study, histopathological examination revealed the presence of spherules embedded in the necrotic tissue, this confirmed that the placental tissue is a favorable environment for growth of *coccidioides*.In this respect ,it was reported that the placenta contains substances which enhance fungal growth. (10).

Detection and identification of these pathogens was also performed using PCR. (11)

In addition, several previous reports have described the identification of*Coccidioides* spp. by PCR, either from environmental sources (12), clinical isolates (13), or clinical specimens (14). Conventional PCR technology, a method which possesses limited utility as a diagnostic tool due to its being an open system and therefore having an increased potential for contamination events was adopted by 14 and 15. Binniker *et al,* have described the development of a real-time PCR assay based on the Light Cycler direct detection of *Coccidioides posadasii* from clinical specimens. (11)

These studies determined the RT-PCR assay to be highly sensitive and specific in the detection of *coccidioides* spp.In the present study, this technique was used to confirm the results ofhistopathology and microbiology.

The efficiency of the procedure was evident in determining the species of coccidiodes infection to be *C. posadasii* upon the use of specific primers, a result which could not be reached by histopathological or microbiological examination alone. Boiling for15 to 20 min has been reported as a safe procedure to inactivate suspensions of mycelia from *Coccidioides* spp. (15).

For the safe extraction, a procedure was described by Burt *et al.*whereby, liquid cultures of *Coccidioides* spp. were frozen in liquid nitrogen and then lyophilized before DNA was extracted.(16) In the present study, inactivation was achieved by boiling which did not negatively affect the results of DNA extraction.

This study has shown the importance of *C. posadasii* as a causative agent of

mycotic placentitis and abortion which is regarded as a major source of herd as well as economic losses that should be efficiently treated.

Reference:

Anderson, ML. (2007)..Infectious causes of bovine abortion during mid- to late-gestation.Theriogenol,; 68:474-486.

Jensen, HE,.Basse,AandAalbaek, B .(1989).Mycosis in stomach compartiments of cattle .ActaVeterinaria Scandinavica,30:409-423.

Garcia,M.E.,Caballero,S.Alvarez-perezand Blanco, J.L.(2008). Seroprevalence of Aspergillus fumigatus Antibodies in bovine herds with a history of reproductive disorders. Veterinarni Medicina,53:117-123

.Ali,R. and.Khan, I.H .(2006).Mycotic abortion in cattle. Pakistan Vet,J.,26:44-46.

Kawazu,M., Kanda, Y. Nannya, K. Aoki, M. Kurokawa, S.Chiba,T.Motokura,H.Hurau and Ogawa, S. (2004). Prospective comparison of the diagnosis potential of Real-Time PCR, Double-Sandwich Enzyme-Linked Immunosorbent Assay screening for Invasive Aspergillosis in patients with hematological disorders.J.Clin.Microbiol.,pp:2733-2741.

Mennink-Kersten, M.H.S.H., Donnelly J.P. and Verweij, P.E. (2004). Detection of circulation galactomannan for the diagnosis and management of invasive aspergillosis. Lancet,Infect.Dis .,4:349-357.

Jenson ,H.E. 1993.Crossed immunoelectroforesis of fungal antigens in tissues as a means of diagnosing systemic aspergillosis and zygomycosis in catlle .Veterinary Research communication ,17:267-275.

8. Knudtson, W.U.; Kirkbride, C.A.(1992).Fungi associated with bovine abortion in the northern plains states (USA) . J .Vet. Diagn. Invest. 4:181-185

9. Abd El-Razik,K.A. ;Ahmed ,Y.F.Soror A.H and Danial, E,N .(2011).Diagnosis of some mycoticplacentitis in small ruminant using PCR in formalin fixed paraffin embedded tissues .Journal .of biotechnology .and biochemistry 6 (2) :72-78

10.El-Nagger, A.L.;.Ahmed,F.A.; Ibrahim and Refai , M.K.M.(1997).Mycotic abortion in small Ruminant, induced by *Aspergillus fumigatous* in Egypt .EygptJ.Comp.Pathol and Clinipathol 10:59-76.

11. Binnicker, M.J; Buckwalter, S.P. and Eisberner, J.J.(2007). Detection of *coccidioides species* in clinical specimens by real-time PCR. J. Clin. Microbiol; 45:173-8

12.Greene, D. R., G. Koenig, M. C. Fisher, and Taylor. J. W. 2000. Soil isolation and molecular identification of *Coccidioidesimmitis*. Mycologia 92**:**406–410.

13. Bialek, R., J. Kern, T. Herrmann, R. Tijerina, L. Cecenas, U. Reischl, and Gonzalez,G. M.(2004). PCR assays for identification of *Coccidioidesposadasii* based on the nucleotide sequence of the antigen 2/proline-rich antigen. J. Clin. Microbiol. 42:778–783

14.Johnson, S. M., K. A. Simmons, and Pappagianis. D.(2004). Amplification ofcoccidioidal DNA in clinical specimens by PCR. J. Clin. Microbiol. 42:1982– 1985.

157.Valesco, M., and Johnston. K. (1997). Stability of hybridization activity of *Coccidioides immitis*in live and heat-killed frozen cultures tested by Accu- Probe *Coccidioides immitis*

culture identification test. J. Clin. Microbiol.
35:736–737.
16. Burt ,A ;Carterb,G.; Koenig ,T. and Taylor, W. (1995). A safe method of extracting DNA from *coccidioides immitis* .Fungal Genet.Newsl .42:23