Global Journal of Biotechnology & Biochemistry 6 (2): 72-78, 2011
ISSN 2078-466X

Diagnosis of Some Mycotic Placentitis in Small Ruminants Using PCR in Formalin-Fixed Paraffin Embedded Tissues

[1]*Khaled A. Abd El-Razik,* [1]*Youssef F. Ahmed,*
[1]*Ashraf H. Soror and* [2]*Enas N. Danial*

[1]Department of Animal Reproduction & AI, National Research Center, Giza Egypt
[2]Department of Chemistry Natural and Microbial Products. National Research Center Giza, Egypt

Abstract: Diagnosis of diseases depends on histopathology using formaldehyde-fixed, paraffin-embedded tissue. Alternative handling methodologies such as Polymerase Chain Reaction (PCR) helps the pathologists for confirmative diagnosis of many diseases including mycotic infectious diseases. The present study was carried out on pregnant sheep and goats experimentally infected with *A.fumigatus* and *C.albicans*. Formaldehyde-fixed, paraffin-embedded placental tissues were stained with haematoxylin & eosin (HES), periodic acid Schiff (PAS) and Gomori methanamine silver (GMS) stains for histopathological examination, followed by PCR using specific primers for *A. fumigatus* and *C. albicans*. Histopathological examination of placental tissue from aborted ewes and goats due to *A. fumigatus* revealed extensive necrotizing placentitis with most of the necrotic area and blood capillaries massively invaded by *A. fumigatus* hyphae. In case of *C.albicans* aborted animals, placenta revealed severe extensive necrotic placentitis with *C. albicans* spores scattered in the affected area and pseudohyphae were penetrating the vascular and fetal membranes. All Aspergillus hyphae and candida pseudohyphae gave positive results with PAS and GMS stain. Using PCR, confirmed the histopathological results as it gave positive results with all these experimentally infected animals. This technique showed high sensitivity in formalin fixative, paraffin placenta tissue blocks although there were no pre-treatment steps in the DNA extraction procedure. This technique was also highly specific to identify the mycotic agents to genus level. In conclusion, histopathological changes accompanied with PCR on the fixed tissue samples are essential for sensitive and accurate diagnosis of mycotic placentitis in small ruminants.

Key words: Placental infection · *Aspergillus fumigatus* · *Candida albicans* · PCR · Histopathological techniques

INTRODUCTION

Mycotic placentitis is a major worldwide cause of abortion in animals. It tends to sporadically occur although on some occasions a significant percentage (10-20 %) of pregnant animals in a herd may be affected. It usually occurs in the third trimester of pregnancy [1].

Aspergillus fumigatus is a ubiquitous fungus which is responsible for gastrointestinal infections, pneumonia and mammary gland infections, both in humans and in animals [2] and considered the most common cause of mycotic placentitis [3]. Also, it is responsible for about 75% of mycotic abortions [4,5]with most of the remaining 25 % being caused by *Zygomycetes* and *Candida spp.* [4, 5].

The hematogenous spread of the fungus may be exacerbated by any immunodeficient or immunosuppressive condition of the host such as corticosteroid therapy, prolonged antibiotic treatment, local treatment of vagina and cervix, infection with viruses such as BVDV and IBR [6] and metabolic disturbance or stress factors [7-9].

Diagnosis of mycotic abortion is mainly carried out using microscopical, serological and cultural examinations [10]. The microbiological methods are time-consuming and lack sensitivity, while the serological tests have variable sensitivity and specificity [11,12]. Therefore, histopathological examination is essential to confirm the mycotic infection from the morphological details of fungi within tissue sections, but as the appearance of hyphae

Corresponding Author: Khaled A. Abd El-Razik, Department of Animal Reproduction, National Research Center, Cairo, Egypt. E-mail: khaledemara707@yahoo.com.

in sections is altered by a number of factors, this may be considered difficult for some investigators [13].

In view of the lack of sensitivity and specificity of current diagnostic methods, research continues to find a methodology which would allow an early and effective diagnosis of the mycotic disease [9]. Recently, techniques based on molecular biology have been adapted for the identification of mycotic pathogens [14,15]. Such techniques provide early, simple, sensitive and reliable diagnosis of these infections [16,17].

During recent years, PCR methods performed with deparaffinized tissue sections have been used to improve the detection and identification of pathogens in fixed specimens [18,19]. Therefore, the present study reported the profits of the PCR method performed with embedded paraffin fixed tissue in some cases of placentitis due to *Aspergillus fumigatus* and *Candida albicans* infections in small ruminants.

MATERIALS AND METHODS

- Systemic mycotic abortion was induced in pregnant ewes and goats in two separated previous studies [20, 21]. In the first experiment, groups of pregnant ewes and goats were intravenously inoculated with 10 ml of viable spore suspension of *Aspergillus fumigatus*, while in the other study the same was done using *Candiada albicans*.
- Archived blocks of paraffin section from these animals were used for both histopathological examination and PCR assay.

Histological Examinations: Paraffin wax sections 5 μm thicknesses were stained with Haematoxylin & Eosin (HES), Periodic Acid Schiff (PAS), Gomori Methanamine Silver (GMS) and examined for *A. fumigatus* and *C. albicans* [22].

Molecular Diagnosis: Paraffin sections of the same tissue blocks were subjected to molecular diagnosis of mycotic placentitis using PCR.

Polymerase Chain Reaction (PCR)

Reference Strains Preparation: *A. fumigatus* and *C. albicans* reference strains used as positive controls were kindly offered by the Department of Chemistry of Natural and Microbial Products-National Research Center, Egypt. *A.fumigatus* was grown in Sabouraud Dextrose Agar media (Cat.no 211584,Difco Laboratories, Detroit, Mich.) at 25-35°C for 2-5 days, while *C. albicans* was cultured on solid yeast peptone dextrose media (YPD)(Cat.no 242720, Difco Laboratories, Detroit, Mich.)at 30°C for 48 h prior to DNA isolation.

DNA Extraction: DNA was extracted directly with no pre-treatment from a loopful of fungal cells using DNeasy Blood & Tissue Kit (Qiagen Co. Cat. no. 69504) following the manufacturer instruction, while formalin fixed, paraffin embedded placental tissue sections (25mg) were deparaffinized with xylene and DNA was extracted using the same kit according to the manufacturer instructions too.

***A. Fumigatus* Primers and PCR Amplification:** The PCR primers used in this study were designed according to Logotheti *et al.* [23] using the Primer Quest program (http:// www.idtdna.com/ Scitools /Applications/ Primerquest) and corresponded to sequences of *A. fumigatus* aspergillopepsin gene deposited in the GenBank database as shown in table.1

Each PCR reaction mixture (50 μl) contained 25μl PyroStart™ Fast PCR Master Mix (Fermentas Co. Cat. no. Ko211), 2 μl for each primer (100 pmol) with 6μl of DNA extracted from pure fungal culture or 21 μl of DNA extracted from paraffin sections.

PCR was performed in PTC-100 Peltier Thermal Cycler (MJ Research, Incline Village, USA). The first cycle included 1min of denaturation at 95°C. This first step was followed by 39 cycles of 2s of denaturation at 94°C, 5s of annealing at 57°C and 10s of primer extension at 72°C and a final extension step of 72 °C for 10 min. A 10μl aliquot of the amplified product was analyzed on 1% agarose gel and stained with ethidium bromide. Appropriate positive and negative controls were included.

Table 1: *A. fumigatus* and *C. albicans* -specific primer pairs characteristics

	Primer pairs	Sequence (5'-3')	Amplicon size (bp)
A. fumigatus	PEX1	TATGTCTTCCCCTGCTCC	250 bp
	PEX2	CTATGCCTGAGGGGCGAA	
C.albicans	SC1F	CGGAGATTTTCTCAATAAGGACCAC	670bp
	SC1R	AGTCAATCTCTGTCTCCCCTTGC	

***C. albicans* Primers and PCR Amplification:** The oligonucleotide primer pair (Table 1) was designed by Galán *et al.* [17] in corresponding to sequences of *C. albicans* KER1 gene deposited in the Gen Bank database. The PCR mixture and conditions were identical to that of *A. fumigatus* except the annealing at 60°C for 15s.

RESULTS

Microscopical examination of placental tissue collected from aborted sheep and goats experimentally infected with *A.fumigatus* revealed extensive necrotizing placentitis. Coagulative necrosis which was extended throughout foetal and placental tissues of chorioallantoic villi was also detected. The area of necrosis was associated with massive polymorphonuclear cells and lymphocytes (Fig. 1). Most of the necrotic area and blood capillaries were massively invaded by *A. fumigatus* hyphae which gave positive result with PAS (Figs. 1&2) and GMS stain (Figs. 3 &4). The hyphae were branching dichotomously at angle of 45°C, they usually have uniform width and are septated when examined at high magnification.

Histopathological examination of placentome of aborted ewes and goats infected with *C. albicans* revealed severe extensive necrotic placentitis. The necrotic area showed massive infiltration with mononuclear cells, neutrophils and macrophages. Phagocytic activity of trophoblast cells toward *C. albicans* was detected. *C. albicans* spores were

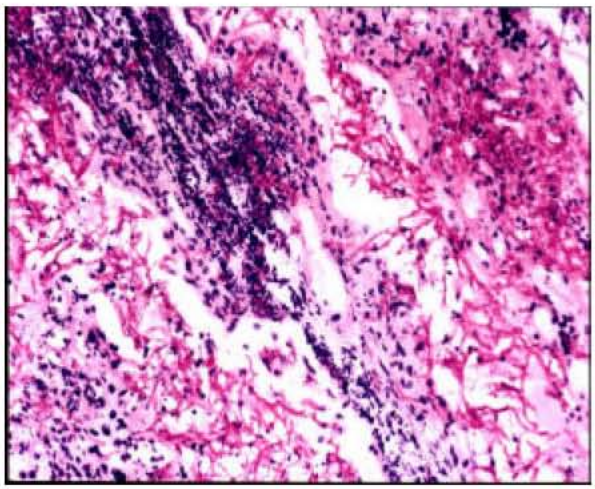

Fig. 1: A section in placenta of experimentally infected she goat with A. fumigatus showing sever degeneration and necrosis of maternal and fetal placenta with mycotic hyphae distributed and associated with neutrophils infiltrations. [PAS stain, X200].

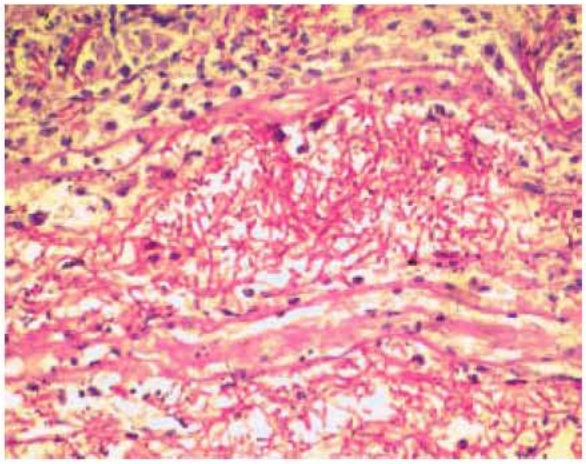

Fig. 2: A section in placenta of experimentally infected sheep with *A. fumigatus* showing

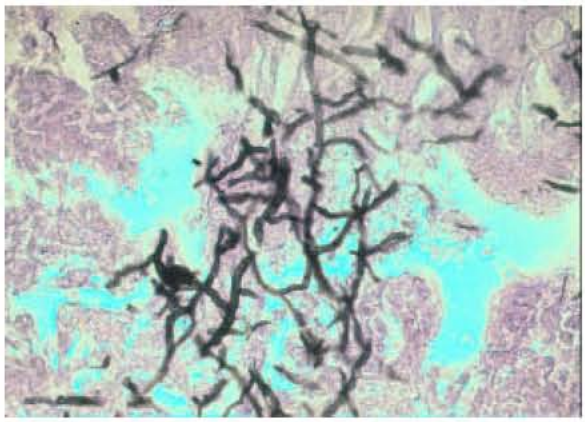

Fig. 3: A section in placenta of aborted goat, showing psudohyphae of *C. albicans*. (GMS, x400

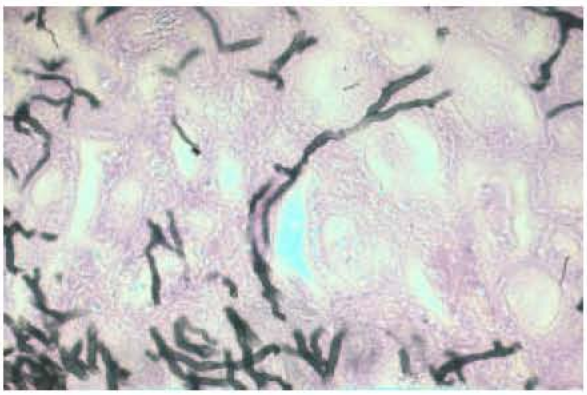

Fig. 4: High magnification of fig.3 (GMS, x800).

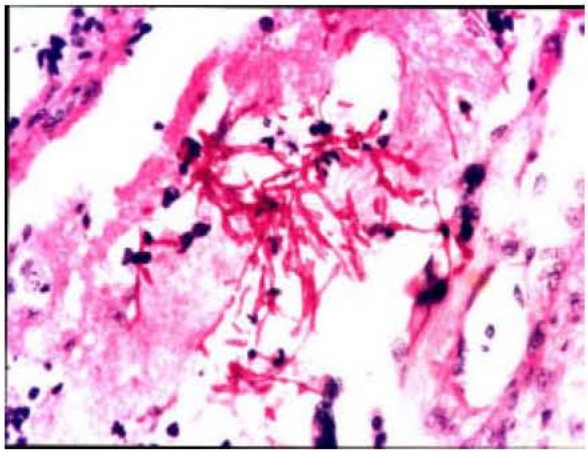

Fig. 5: A section in placenta of aborted goat, showing Pseudohyphae of C. albicans [PAS, X 400]

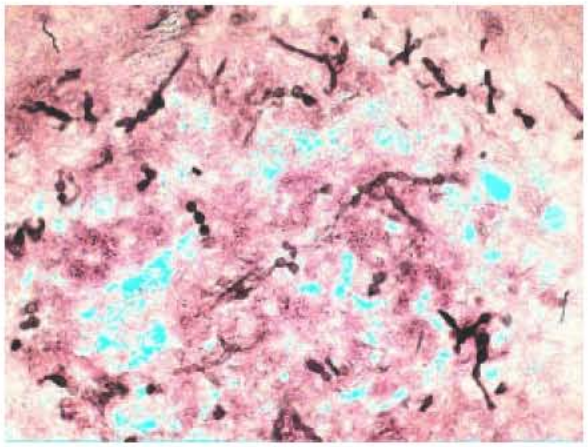

Fig. 6: A section in placenta of aborted goat, showing Pseudohyphae of C albicans [GMS, X 400].

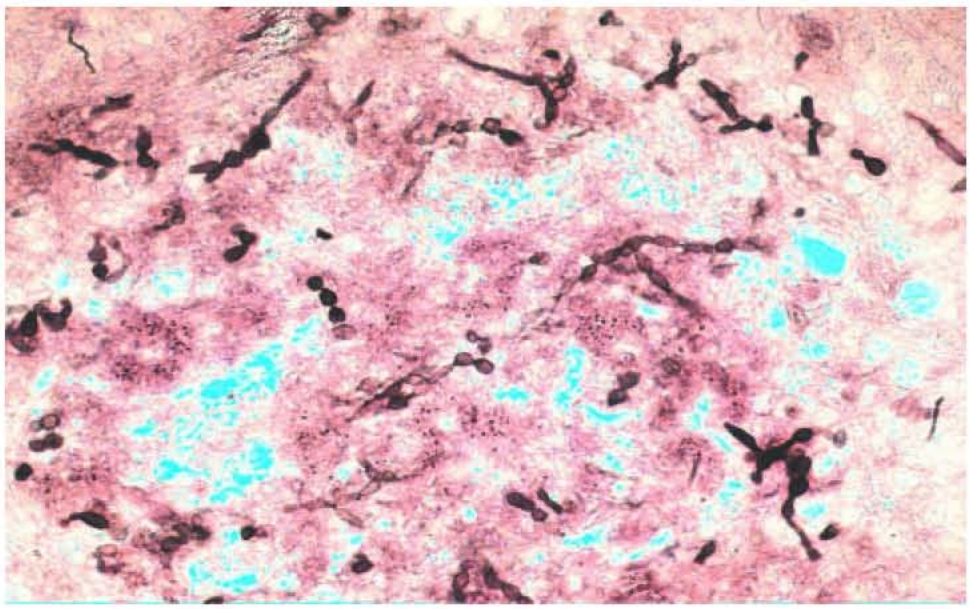

Fig. 7: High magnification of fig.6, showing C.albicans Dimorphism. Blastospores are unicellular forms of the fungus that divide by budding, Hyphal branches and/or secondary branches are produced and constituting a mycelium psudohyphae.

scattered in the affected area and pseudohyphae were penetrating the vascular and fetal membranes and they were positive with PAS (Fig. 5) and GMS stains (Fig.6). Like other pathogenic fungi, *C. albicans* is dimorphic. This yeast exhibited a number of different morphological forms under different environmental conditions; such forms include budding yeast cells (blastospores, blastoconidia), pseudohyphae (elongated cells which appear as filamentous cell chains), true hyphae and clamydospores which were positive with GMS (Fig. 7).

For confirmation of the histopathological examination, PCR was performed using DNA extracted directly with no pre-treatments from formalin fixed, paraffin embedded placental tissues. This technique showed high sensitivity and specificity in the detection and identification(to the genus level) of *A. fumigatus* and *C.albicans* that are responsible for mycotic placentitis in small ruminants as showed in Fig. 8 & 9. PCR gave the expected PCR product in all *A. fumigatus* (250bp) and *C.albicans* (670bp) experimentally infected sheep and goats.

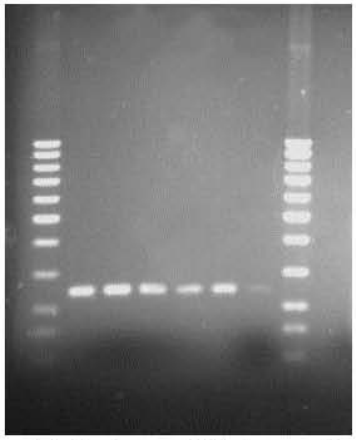

Fig. 8: PCR of *A. fumigatus* DNA in paraffin-embedded tissues. Lanes 1&8,100bp ladder; Lane 2, positive control; Lanes 3-7, amplified *A. fumigatus* DNA(250bp) in aborted fetal tissue samples;Lane9, negative control.

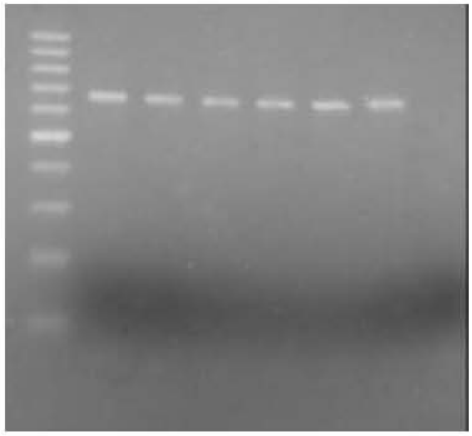

Fig. 9: PCR of *C. albicans* DNA in paraffin-embedded tissues. Lanes 1&8,100bp ladder; Lane 2, positive control; Lanes 3-7, amplified *C. albicans* DNA (670bp) in aborted fetal tissue samples; Lane9, negative control.

DISCUSSION

Fungi can produce reproductive failure in animals either as a direct result of establishing infection in the genital system or by producing toxin metabolites (mycotoxins) [8], which are consequently ingested and absorbed. Mycotic abortion is the most important consequence of fungal infection of the genital tract (6.8%) caused commonly by both *A. fumigatus* (62%) and *C. albicans*(2%) that normally present in the cervicovaginal cavity of dairy cows with or without reproductive diseases [3, 8].

Current diagnostic methods have not proven to be sufficiently sensitive and specific to enable an early and effective diagnosis of the mycotic diseases, with the result that the search for an optimal diagnostic method continues [24]. As fungi are ubiquitous in the environment their presence could be the result of contamination so the diagnosis of mycotic abortion requires compatible lesions in the placenta (necrotizing placentitis) or fetus in addition to the microscopic demonstration or isolation of fungi [7] and the histopathological evaluation as part of a postmortem examination.

From the histopathological examination, it was evident that placental tissues are a favorable environment for growth of *A. fumigatus*. This was in agreement with El-Naggar *et al.* [20]. In this respect, it was reported that placenta contains substances which enhance fungal growth [25]. The high infiltration with polymorphonuclear and lymphocytic cells in the necrosed area indicated that *A. fumigatus* may cause suppurative inflammation. Also, polymorphonuclear cells play a significant role in host defense against Aspergillosis as reported by Diamond and Clark [26]. Hyphae of *A. fumigatus* were massively scattered throughout the necrotic tissue of placentome and concentrated mainly in the maternal placenta directed to the chorionic villi of foetal placenta. These results agreed with El-Naggar *et al.* [20] and Krogh [27]. Regarding *C. albicans*, Necrotizing placentitis in aborted animals may be due to invasion of *C. albicans* into blood vessels and capillaries of maternal and foetal placenta leading to thrombosis and necrosis of affected area as a result of metabolites or toxic products of *C. albicans* hyphae in tissue. Besides, Phagocytic activity of trophoblast cells toward *C. albicans* was detected and pseudohyphae were penetrating the vascular and fetal membranes which were positive with PAS. Similar results were obtained by El-Naggar *et al.* [21].

In recent years, PCR methods performed with deparaffinized tissue sections have been used to improve the detection and identification of pathogens in fixed specimens [19]. This methodology needs to use strict positive and negative controls and exact comparison with histological features and mycological analysis is required. However, false-negative and false-positive results due to artifacts in amplification, or sampling and possible exogenous contamination by microorganisms are still very important pitfalls in molecular diagnostic pathology [28, 29]. In the present study, there was no evidence of contamination by external sources and strict laboratory precautions were applied to avoid carry over and false positive results.

In this work, primers corresponded to sequences of *A. fumigatus* aspergillopepsin gene and *C. albicans* KER1 gene were used for molecular diagnosis of *A.fumigatus* and *C.albicans* directly from formalin fixed paraffin embedded placental tissue blocks of experimentally infected sheep and goats. The PCR techniques used here for confirmation of the results of histopathological examination, showed a high sensitivity in paraffinized tissue of all infected animals in comparison with the positive controls although the extraction procedure didn't involve any pre-treatment steps. This came in contrary with previous reports that necessitate the use of a variety of disruption methods to lyse conidia and hyphae which include freezing in liquid nitrogen, grinding with mortar and pestle, sonication, glass bead milling and microwaving [30-34]. Since earlier detection of infection permits prompt initiation of antifungal therapy with a greater likelihood for improved survival and reduced morbidity, therefore PCR is preferred for confirmation of mycotic placentitis causative agents even in fixed tissues. However, further studies are needed for evaluation of this technique in tissue samples from naturally infected small ruminants with mycotic placentitis.

In conclusion, following histopathological examination, because of the used PCR ability to detect extremely small quantities of DNA, it is a promising mean to confirm mycotic placentitis and abortion and offers many advantages even when applied on archived formalin-Fixed Paraffin embedded tissue.

REFERENCES

1. Tell, L.A., 2005. Aspergillosis in mammals and birds: impact on veterinary medicine. Medical Mycol., 43: S71-S73.
2. Las Heras, A.L., I. Dominguez, M.J. Lopez, L. Paya, F. Pena, L. Mazzucchelli, A. Garcia and J.F. Fernandez-Garayzabal, 2000. Intramammary *Aspergillus fumigatus* infection in dairy ewes associated with antibiotic dry therapy. Veterinary Record, 147: 578-580.
3. Knudtson, W.U. and C.A. Kirkbride, 1992. Fungi associated with bovine abortion in the northern plains states (USA). J Vet Diagn Invest, 4: 181-185.
4. Jensen, H.E. and J. Hau, 1990. A murine model for the study of the impact of *Aspergillus fumigatus* inoculation on the foeto-placental unit. Mycopathologia, 112: 11-18.
5. Sarfati, J., H.E. Jensen and J.P. Latge, 1996. Route of infection in bovine aspergillosis. Journal of Medicine and Veterinary Mycol., 34: 379-383.
6. Jensen H.E., A. Basseand and B. Aalbaek, 1989. Mycosis in the stomach compartiments of cattle. Acta Veterinaria Scandinavica, 30: 409-423.
7. Anderson, M.L., 2007. Infectious causes of bovine abortion during mid- to late-gestation. Theriogenol., 68: 474-486.
8. Garoussi, M.T., A.R. Khosrave and P. Havareshti, 2007. Mycoflora of cervicovaginal fluids in dairy cows with or without reproductive disorders. Mycopathologia, 164: 97-100.
9. Garcia, M.E., J. Caballero, S. Alvarez-Perezand and J.L. Blanco, 2008. Seroprevalence of *Aspergillus fumigatus* antibodies in bovine herds with a history of reproductive disorders. Veterinarni Medicina, 53: 117-123.
10. Ali, R. and I.H. Khan, 2006. Mycotic abortion in cattle. Pakistan Vet. J., 26: 44-46.
11. Kawazu, M., Y. Kanda, Y. Nannya, K. Aoki, M. Kurokawa, S. Chiba, T. Motokura, H. Hurau and S. Ogawa, 2004. Prospective comparison of the diagnostic 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.
12. Mennink-Kersten, M.A.S.H., J.P. Donnelly and P.E. Verweij, 2004. Detection of circulating galactomannan for the diagnosis and management of invasive aspergillosis. Lancet, Infect. Dis., 4: 349-357.
13. Jensen, H.E., 1993. Crossed immunoelectroforesis of fungal antigens in tissues as a means of diagnosing systemic aspergillosis and zygomycosis in cattle. Veterinary Research Communications, 17: 267-275.
14. Kanbe, T., T. Horii, T. Arishima, M. Ozekiand and A. Kikuchi, 2002. PCR based identification of pathogenic *Candida species* using primer mixes specific to Candida DNA topoisomerases II genes. Yeast, 19: 973-989.
15. Trost, A., B. Graf, J. Eucker, O. Sezer, K. Possinger, U.B. Gobel and T. Adam, 2004. Identification of clinically relevant yeasts by PCR/RFLP. J. Microbiol. Methods, 56: 201-211.
16. Arancia, S., S. Sandini, A. Cassone, F. De Bernardis and R. La Valle, 2004. Construction and use of PCR primers from a 65 kDa mannoprotein gene for identification of *C. albicans*. Mol. Cell. Probes., 18: 171-175.
17. Galán, A., V. Veses, A. Murgui, M. Casanova and J.P. Martínez, 2006. Rapid PCR-based test for identifying *Candida albicans* by using primers derived from the pH-regulated KER1 gene. FEMS Yeast Res., 6: 1094-1100.

18. Procop, G.W. and M. Wilson, 2001. Infectious disease pathology. Clin. Infect. Dis., 32: 1589-1601.
19. Procop, G.W., 2007. Molecular diagnostics for the detection and characterization of microbial pathogens. Clin. Infect. Dis., 45: S99-S111.
20. El-Naggar, A.L., Y.F. Ahmed, F.A. Ibrahim and M.K.M. Refai, 1997a. Mycotic abortion in small ruminants induced by *Aspergilus fumigatus* in Egypt. Egypt. J. Comp. Pathol. & Clinc. Pathol., 10: 59-76.
21. El-Naggar, A.L., Y.F. Ahmed, F.A. Ibrahim and M.K.M. Refai, 1997b. Mycotic abortion in small ruminants induced by *Candida albicans*. Egypt. J. Comp. Pathol. & Clinc. Pathol., 10: 77-92.
22. Sheehan, D.C. and B.B. Hrapchak, 1980.Theory and practice of Histotechnology, The C.V. Mosby, St. Louis.
23. Logotheti, M., A. Kotsovili-Tseleni, G. Arsenis and N.I. Legakis, 2009.Multiplex PCR for the discrimination of *A. fumigatus*, *A. flavus*, *A. niger* and *A. terreus*. J. Microbiological Methods, 76: 209-211.
24. Shokri, H., A. Khosravi, A. Sharifzadeh and Z. Tootian, 2010. Isolation and identification of yeast flora from genital tract in healthy female camels (*Camelus dromedarius*). Veterinary Microbiol., 144: 183-186.
25. Gorbel, M.J. and S.M. Eaded, 1973. The effect of soluble extract of bovine placenta on the growth of fungi implicated in bovine mycotic abortion. Br. Vet. J., 129: 75-79.
26. Diamond, R.D. and R.A. Clark, 1982. Damage to *Aspergillus fumigatus* and *Rhizopusoryzae* hyphae by oxidative and non -oxidative microbial products of human neutrophils in vitro. Inf. Immun., 38: 487-495.
27. Krogh, H.A., 1985. Bovine mycotic abortion in Denmark. Nord. Vet. Med., 37: 27-33.
28. Catalouk, O., E.A. Cakmak, N. Buyukberber, O. Barlas, 2003. Formalin fixing and paraffin embedding may lead to extra band development in PCR. New Microbiol., 26: 193-8.
29. Mies, C., 1994. Molecular biological analysis of paraffin-embedded tissues. Hum Pathol., 25: 555-60.
30. Haugland, R.A., N. Brinkman and S.J. Vesper, 2002. Evaluation of rapid DNA extraction methods for the quantitative detection of fungi using real-time PCR analysis. J. Microbiol. Methods, 50: 319- 323.
31. Jin, J., Y.K. Lee and B.L. Wickes, 2004. Simple chemical extraction method for DNA isolation from *Aspergillus fumigatus* and other *Aspergillus* species. J. Clin. Microbiol., 42: 4293- 4296.
32. Maaroufi, Y., N. Ahariz, M. Husson and F. Crokaert, 2004. Comparison of different methods of isolation of DNA of commonly encountered *Candida* species and its quantitation by using a real-time PCR-based assay. J. Clin. Microbiol., 42: 3159- 3163.
33. Millar, B.C., X. Jiru, J.F. Moore and A. Earle, 2000. A simple and sensitive method to extract bacterial, yeast and fungal DNA from blood culture material. J. Microbiol. Methods, 42: 139- 147.
34. Williams, R.H., E. Ward and H.A. McCartney, 2001. Methods for integrated air sampling and DNA analysis for detection of airborne fungal spores. Appl. Environ. Microbiol., 67: 2453- 2459.