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APPLICATION OF REAL-TIME PCR FOR DETECTION OF *ASPERGILLUS* SPECIES IN ABORTED RUMINANT FOETUSES

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Summary

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This research has performed for detection of *Aspergillus* species (*A. fumigatus*, *A. flavus*, *A. niger* and *A. terreus*) in aborted bovine, ovine, caprine and camel foetuses by real-time PCR in Iran. After modification of real-time PCR on abomasal contents, from the total number of 970 samples, 141 (14.53%) gave positive results for *Aspergillus* species. Of them, 62 (17.71%), 33 (14.04%), 27 (12.05%) and 19 (11.8%) positive specimens were detected in bovine, ovine, caprine and camel foetuses respectively. Statistical analysis showed significant differences ($P<0.05$) between bovine and camel and bovine and caprine aborted foetuses. *Aspergillus* abortion was the most prevalent in cattle whereas camels tended to be the most resistant. This study was the first report of direct identification of *Aspergillus* species by real-time PCR in aborted bovine, ovine, caprine foetuses in Iran and camel foetuses in the world.

Key words: aborted foetuses, *Aspergillus* spp., real-time PCR

INTRODUCTION

There are many causes of abortion but unfortunately despite the progress of science, in half of cases there is no certain known reason for animal abortion. Infectious bovine rhinotracheitis (IBR) (Muylkens *et al.*, 2007), leptospirosis (Thiermann, 1982), brucellosis (Şahin *et al.*, 2008) and mycoses (Tell, 2005) are the main cause of abortion in bovine between the 6th to 9th months of pregnancy.

Mycotic abortions are a consequence of ingestion or inhalation of moulds grown on poorly preserved feeds by animals. Their incidence is usually seasonal with a peak between November and April (Ainsworth *et al.*, 1955).

Aspergillus spp. are common and widespread animal pathogens causing a number of different diseases, including allergic disease, saprophytic disease, mastitis and abortion.

Systemic aspergillosis is difficult to diagnose clinically because there is no typical clinical picture and therefore most cases are not diagnosed until post-mortem examination. The treatment of mycoses, including aspergillosis, is challenging as most agents are resistant to commonly used azole drugs such as itraconazole (Denning *et al.*, 1997).

Conventional diagnostic methods such as culture and galactomannan assays are

not recommended for mycoses detection as they lack sensitivity and specificity, are time-consuming and labour-intensive (Denning, 1998; Latge, 1999).

It should be emphasized that the growth of *Aspergillus* shown by identification of fungal colonies in culture media is very difficult to be interpreted as truly etiological (Jensen *et al.*, 1996) as false positive results may occur due to the growth of *Aspergillus* on the foetus following post-abortion contamination.

Polymerase chain reaction (PCR) detection of *Aspergillus* DNA is increasingly used for the non-culture based diagnosis of *Aspergillus* in available samples, without recourse to invasive procedures (Alexander & Pfaller, 2006). Recently, the real-time PCR was described as a fast, accurate and sensitive analytical method for identifying and quantifying moulds even to the species level (Costa *et al.*, 2002). Over the years, various PCR methods including conventional PCR (Van Burik *et al.*, 1998), competitive PCR, (Bretagne *et al.*, 1995), nested PCR (Buchheidt *et al.*, 2004), quantitative PCR (qPCR) (Bowman *et al.*, 2001), real-time qPCR (Costa *et al.*, 2001), PCR-enzyme linked immune sorbent assay (PCR-ELISA) (Hadrich *et al.*, 2011) and real-time PCR (Trama *et al.*, 2005) have been used for detection of *Aspergillus* DNA in clinical specimens and in all of these studies, the PCR assay is introduced as an accurate, sensitive, specific and fast assay for detection of mycoses.

The PCR analysis has been developed for the detection of *Aspergillus* in a wide variety of clinical samples such as bronchoalveolar lavage fluid (White *et al.*, 2010), serum (Badiee *et al.*, 2009), soil (Hong *et al.*, 2010), indoor environment (Udagawa *et al.*, 1996), blood (Hummel *et al.*, 2010), sputum and brain (van der Linden *et al.*, 2010) and bone marrow bioptate (Badiee *et al.*, 2010).

The present study was carried out to detect *Aspergillus* spp. in aborted bovine, ovine, caprine and camel foetuses by evaluation of real-time PCR.

MATERIALS AND METHODS

Samples

From November to April of 2010, a total of 970 aborted foetuses including 350 bovine, 235 ovine, 224 caprine and 161 camel were collected from 953 commercial dairy herds from various parts of Iran. Samples of abomasal contents of aborted foetuses were collected and sent immediately under refrigeration to the laboratory. Samples were stored at –20 ^{o}C until DNA extraction.

DNA extraction

DNA was extracted from abomasal contents of aborted foetuses by using DNA extraction and purification kit (Invitrogen, Paisley, U.K.) according to manufacturer's instruction. The total DNA was measured at 260 nm optical density. The real-time PCR, and amplicon detection were performed in separate rooms, to avoid contamination by amplicons.

Real-Time PCR

Aspergillus fumigatus (*A. fumigatus*) mitochondrial transfer RNA -Thr, -Glu, -Val, -Met and ribosomal RNA gene (GenBank L37095) was used for amplification as published earlier (Bolehovska *et al.*, 2006), with the amplicon size 91 bp. Real-Time PCR for *Aspergillus* DNA detection was modified in Islamic Azad University, Shahrekord branch's laboratory.

Amplification and on-line quantification was carried out on a LightCyclerR 1.5 Instrument (Roche Diagnostics, Mannheim, Germany). Two hybridization probes were used (BioNeer Corporation, South Korea): 24-mer 5'-CTGTTA GTGCGGGAGTTCAAAXTCT-3', where X is the internal labelling of T by the LightCycler (LC) Red 640, and 28-mer 5'-CTGAGCTAATTTCTTTCAACCCA AGGGA-3', labeled at the 3'end by fluorescein isothiocyanate.

The LC Red 640 hybridization probe also served as sense primer for template amplification. The 22-mer antisense primer sequence was 5'-AACACC TGACCTTTCGCGTGTA-3'. The PCR mixture of 10 μL in volume consisted of 1× LightCycler Fast-Start DNA Master HybProbe (Roche Diagnostics, Mannheim, Germany), 3 mmol/L $MgCl_2$, 0.2 U UNG (uracil-DNA-glycosylase, Roche Diagnostics, Mannheim, Germany), the respective primer in the concentration of 0.8 μmol/L, the fluorescein isothiocyanate-labelled probe in concentration of 0.4 μmol/L, and 5 μL of the template. The temperature profile of the PCR reaction was as followed: 50 °C for 5 min for UNG activation, 95 °C for 10 min as the initial denaturation, and 55 cycles with the temperatures of 95 °C for 5 s, 60 °C for 10 s, and 72 °C for 15 s.

Sample fluorescence was measured in each cycle at the end of the annealing. In order to verify the absence of DNA polymerase inhibitors, end-point PCR followed by agarose gel electrophoresis was carried out. Part of the gene for the human beta-globin was used as an internal control of the amplification.

Statistical analysis

Data were transferred to Microsoft Excel spreadsheet (Microsoft Corp., Redmond, WA, USA) for analysis (SPSS 18.0 statistical software, SPSS Inc., Chicago, IL, USA). Differences were considered significant at $P<0.05$.

RESULTS

The real-time PCR reaction was positive with examined strains *A. fumigatus* AF13, *A. flavus* ATCC 36607, *A. niger* ATCC 10535, *A. terreus* DSM 826. A total of 141 out of 970 aborted foetuses (14.53%) were reported positive by real-time PCR for presence of *Aspergillus* spp. in aborted bovine, ovine, caprine and camel foetuses in Iran.

In this study, 62 (17.71%), 33 (14.04%), 27 (12.05%) and 19 (11.8%) out of 350, 235, 224 and 161 samples were reported positive for presence of *Aspergillus* spp. in aborted bovine, ovine, caprine and camel foetuses respectively (Table 1). The statistical analysis showed significant differences ($P<0.05$) only bet-

Table 1. Prevalence of *Aspergillus* spp. in aborted bovine, ovine, caprine and camel fetuses in Iran detected by real-time PCR.

Species	Number of samples	Positive by real-time PCR, number (%)
Bovine	350	62 (17.71)
Ovine	235	33 (14.04)
Caprine	224	27 (12.05)
Camel	161	19 (11.80)
Total	970	141 (14.53)

ween cattle and camels and cattle and goats for presence of *Aspergillus* spp.

According to the results of this study, camels appeared to be the most resistant and cows – most sensitive to abortion caused by *Aspergillus* spp.

DISCUSSION

The real-time PCR has some advantages compared to the conventional PCR; it is an important diagnostic tool yielding reliable and reproducible results, does not require post-PCR analysis (gel electrophoresis, hybridization), and the risk of cross contamination is limited.

Previous studies showed that the sensitivity of real-time PCR (79%) was higher than that of ELISA (58%) (Kami *et al.*, 2001) and was comparable to that reported for nested PCR (Kawamura *et al.*, 1999). In the other hand, detection of fungi by real-time PCR is important in determining the appropriate antifungal therapy because the successful outcome depends on accurate and early diagnosis.

To the author's knowledge, there is no significant study about the prevalence of *Aspergillus* in aborted ruminant foetuses. A previous study showed that *A. fumigatus* was the most commonly diagnosed casual organism (Jensen *et al.,* 1993). Another study indicated that the chief fungus associated with mycotic abortion was *Aspergillus fumigatus*, which has been recorded from over 60% of cases (Ali & Khan, 2006). It was also established that *A. fumigatus* was the most pathogenic *Aspergillus* in aborted bovine foetuses (Sarfati *et al.*, 1996). In another study, Jerret *et al.* (1984) mentioned that *Aspergillus* spp. is the commonest cause of mycotic abortion in the area under survey. Several studies from the USA (Hillman, 1969), Germany (Wiekel, 1965) and Australia (Jerrett *et al.*, 1984) indicated that total prevalence of mycotic abortions in cattle was 2–20%. Unfortunately, there is no study about the prevalence of *Aspergillus* spp. in aborted ovine, caprine and camel foetuses and this investigation is the first prevalence report with direct detection of *Aspergillus* spp. in the foetuses of these animals without further evaluation of the species involved.

The prevalence of *Aspergillus* spp. in bovine aborted foetuses of this study (17.71%) was higher than that reported in northern plains states of the USA (5%) (Knudtson & Kirkbride, 1992), South-Eastern Australia (17%) (McCausland *et al.,* 1987) but was lower than in England (20%; Hugh-Jones *et al.*, 1967).

Previous studies suggest that contact with soil and bedding material (Knudtson & Kirkbride, 1992), bovine viral diarrhoea virus causing immunosuppression in cattle by interfering with neutrophil function (Kirkbride, 1985), frequent treatment with steroids and cytotoxins (Rippon, 1988), increase production of cortisol by the foetus prior to parturition (McCausland *et al.*, 1987) are among the factors predisposing to mycotic abortions. Probably due to higher populaton density of cattle in farms, the use of concentrates and grain for cattle's feed, the higher resistance of camels in poor environmental condition and finally camels habitat, cattle were more sensitive to mycotic abortions than camels. Camels usually live in the arid and waterless area. In these conditions, the growth and proliferation of fungi is very slow and therefore mycotic abortions are more rarely encountered, as observed in this study.

In conclusion, our study showed that *Aspergillus* abortions in cattle, sheep, goats and camels in Iran could be notable.

To our knowledge, this study is the first report of direct identification of *Aspergillus* spp. by real-time PCR in aborted bovine, ovine, caprine foetuses in Iran and camel foetuses in the world. The real-time PCR used in this study is an excellent alternative to current sampling strategies. It allows correct detection of four *Aspergillus* spp. (*A. fumigatus, A. flavus, A. niger* and *A. terreus*) and can simplify the procedure by testing presumptive *Aspergillus* genome taken directly from abomasal contents of aborted foetuses.

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