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Early Detection of *Aspergillus fumgitus* in a High Risk Group of Dairy Cattle using Nested and Real-Time Quantitative PCR

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Abstract: Early detection of *Aspergillus fumigatus (A. fumigatus)* may facilitate early diagnosis and prevention of Invasive Aspergillosis (IA) in dairy cows. This study used a nested PCR and real-time quantitative PCR method to measure *A. fumigatus* in feed, tissue and blood samples collected from domestic dairy farms in China. In total, 396 feed samples from across China including corn, soybean meal, cotton seed meal, wheat mill run, distiller's dried grains, corn and grass silage and Total Mixed Ration (TMR) were tested. Of these, 95% contained detectable levels of *A. fumigatus*. Blood and tissue samples from the GI tract of 7 unhealthy cows all tested positive for *A. fumigatus*. The analytical sensitivity of the PCR method was <1 fg/assay.

INTRODUCTION

A. fumigatus is one of the most ubiquitous of the airborne saprophytic fungi. While most individuals are immune to an *A. fumigates* infection, immunosuppression and stress predispose dairy cattle to an infection and systemic mycosis (Latge, 1999; Pellegrino *et al*., 2013). With the increase in the number of immuno suppressed dairy cows and the degree of severity of modern management (Gordon *et al*., 2013; VanLeeuwen *et al*., 2011), *A. fumigates* infection has be come common and can cause severe, often fatal, disease in immunocom promised hosts (Denning, 1998; Chotirmall *et al*., 2013).

Early diagnosis and initiation of antifungal therapy are essential to the health of dairy herds (Zhao *et al*., 2001). Traditionally, the identification of *A. fumigatus* contamination was based on morphological methods which required adequate growth for evaluation of colony characteristics and microscopic features. The limitations of culture, histological and serological techniques have led to the search for more sensitive, non-culture-based methods. The majority of nucleic acid-based fungal detection and identification systems are PCR-based. With the development of PCR techniques, molecular approaches have been used for the detection of *A. fumigatus* from environmental and clinical samples (Lengerova *et al*., 2011; Babouee *et al*., 2013; Zhao *et al*., 2013). As an adaptation of standard PCR, Bowman *et al*. (2002) provided the first description of a method for real-time PCR-based quantitation of *A. fumigatus* tissue burden in an animal model of infection. Quantitative 'real time' PCR systems represent the most recent advance in diagnostic mycology. This method has the potential to not only substantially increase the sensitivity of PCR but also to provide the means to monitor response to therapy by

measuring fungal burden at any given point in time. Several advantages of real-time quantitative PCR assay include: it is a highly sensitive assay, it is performed in a closed-tube system and requires no post-PCR manipulation of the sample, thereby increasing sample throughput and it supports the use of a normalization gene for quantitative PCR or house keeping genes for quantitative RT-PCR controls. Finally, quantitative information on fungal contamination in feed and fungal infection in dairy cows is necessary for the management of herd health and dairy production (Pille *et al*., 2014; Salas *et al*., 2013). We therefore, developed a rapid and sensitive assay, based on real-time PCR technology, for the identification of *A. fumigatus* contamination in a variety of feed and tissue types.

MATERIALS AND METHODS

Feed sample collection: Although *A. fumigatus*is reported as a "ubiquitous mold", data on its prevalence in dairy cow feeds in China had not, before now, been collected. About 396 feed samples from15 dairy farms in Central China were collected. Samples included corn, distillers dried grains, soybean meal, wheat mill run and TMR it is total mixed ration. The samples were transported well-dry samples or wet samples with ice-bag overnight to the lab. DNA was extracted as described as below.

Tissue sample collection: The 7 two-year old Holstein cows from Jilin Agri. University Dairy were chosen for quantitative real-time PCR assay, five which had recently aborted and two which had died suddenly from non-GI-related problems. The samples included cow blood, feed, GI wall, GI content, mesenteric lymphoid node and cotyledon. DNA was extracted as described as below.

Standard *A. fumigatus* genomic DNA: *A. fumigatus* colonies were obtained from the Department of Food Sciences, Jilin Agri. University, Changchun, China. *A. fumigatus* genomic DNA was extracted from the *A. fumigatus* cultureusing QIAGEN QIAmp DNA mini kit following the instructions of manufacturer (QIAGEN Inc., Valencia, CA).

Genomic DNA extraction for PCR analyses: DNA was extracted from blood, GI contents, tissues and feeds using the QIAGEN QIAmp DNA mini kit according to the manufacturer's instructions (QIAGEN Inc., Valencia, CA). The "tissue method" was used for all extractions including blood. With this method, 25 mg of tissue or feed or 25 μL of whole blood or GI contents were processed. DNA was eluted from the QIAGEN column with 200 μL purified water and then stored at -80°C until analysis. All samples were further purified using a spin column (BD Bioscience, Palo Alto, CA) to remove potential contaminants that inhibit the polymerase chain reaction.

PCR-based assay for *A. fumigatus*: This assay was based on the sequences of fungal ribosomal genes. Three ribosomal genes (18S, 5.8S and 28S, respectively) border two variable Internally-Transcribed Spacer (ITS) domains. The sequences within the ribosomal genes are highly conserved however, sequences within the ITS-1 and -2 regions are not conserved and allowed design of primers which specifically bind to *A. fumigatus* genomic DNA (Suarez *et al*., 2008). Extracted DNA was amplified using a thermocycler (Ericomp Power Block II, San Diego, CA). The primers and PCR conditions used are described below.

Two-step nested PCR: A two-step nested PCR was used to confirm the amplicon from step one. The universal primers used for fungal amplification were F1 5'-GGATGTATTTATTAGATAAAAAATCA-3' and R1, 5'-CAGTAGTTAGTCTTCAGTAA-3' which yielded a 743 bp PCR product. A 50-μL PCR mixture containing 10 μL of DNA template, 6 μL of 25 mM $MgCl_2$, 5 μL of PCR buffer without $MgCl_2$, 200 μM each deoxynucleotide triphosphate, 25 pmol of each primer and 1 U of Taq DNA polymerase (Promega, Madison, WI) was used for each reaction. Reactions involved 1 cycle at 95°C for 5 min, followed by 35 cycles with a denaturation step at 95°C for 30 sec, an annealing step at 55°C for 1 min and an extension step at 72°C for 1 min, followed by 1 cycle at 72°C for 6 min.

After the first-round or primary PCR, we used an additional internal primerset for the subsequent second-round or nested PCR to increase the sensitivity and specificity of the assay. For the second amplification, the primers used were *A. fumigatus*-specific: F2, 5'-ATGCCCTTCGGGGCTCCT-3' and R2, 5'-CTGGTTCCCCCCAC-3' which produced a 520-bp PCR product. Nested PCR amplification mixtures contained 1 μL of first-roundproduct in 50 μL of PCR reaction mixture (6 μL of 25 mM $MgCl_2$, 5 μL of PCR buffer without $MgCl_2$, 200 μM each deoxynucleoside triphosphate, 25 pmol of each primer and 1 U of Taq DNA polymerase (Promega, Madison, WI). Reactions involved1 cycle at 95°C for 5 min, followed by 30 cycles with a denaturation step at 95°C for 30 sec, an annealing step at 55°C for 30 sec and an extension step at 72°C for 30's, followed by 1 cycle at 72°C for 6 min.

Quantitative PCR analysis of *A. fumigatus* DNA: A real-time quantitative PCR assay was used to quantify *A. fumigatus* genomic DNA. The primers were designed based on the *A. fumigatus*-specific sequence in the ITS2

highly variable area after comparison with the nucleotide sequences of *A. flavus*, *A. nidulans*, *A. niger*, *A. terreus* and *A. ustus* to assure that no sequence overlap existed.

A forward primer (F3: 5'-ACCTCCCACCC GTGTCTATC-3') and a reverse primer (R3: 5'-GCGG CCGTCGAAACG-3') were synthesized at the Oregon State University Center for Gene Research and Biotechnology and diluted in water. For each reaction, a 1 μL template DNA was combined with 2.5 μL Sybr Green buffer, 3 μL $MgCl_2$ (25 mM), 2 μL of dNTPs (2.5 mM each), 0.125 μL of AmpliTaq (5U μL^{-1}), 0.25 μL of AmpErase (1 U μL^{-1}), 1.5 μL of each primer (50 and 300 nM for the forward and reverse primers, respectively) and 13.13 μL of water (final volume was 25 μL). Concentration of 150 and 600 nM of forward and reverse primers were established in earlier studies, these concentrations minimized but did not eliminate, primer dimerization. A master mix which contained all components of the reaction mixture except template DNA was prepared to minimize variability across samples. A MyiQ™ Single color real-time PCR detection system was used to amplify *A. fumigatus* ITS-1 DNA. The thermocycling program consisted of 45 cycles of denaturing at a temperature of 95°C and an annealing and extension temperature of 60°C.

Absolute quantification: In the real-time PCR, the concentration of *A. fumigatus* was calculated using absolute quantification. A standard curve of genomic template DNA was conducted in all assays. The 200 μL H_2O was used to recover each DNA sample during the DNA extraction and 1μL of this solution was used in the PCR assay. Genomic units were calculated based on the assumption that each spore of *A. fumigatus* contains 29 Megabase pairs of DNA (Nierman *et al*., 2005; Dean *et al*., 2005). Accordingly, in 1 μL blood sample, we may detect 1pg of *A. fumigatus* DNA. We used 200 μL H_2O to recover the DNA sample during the DNA extraction and used 1 μL of this solution in the PCR. We know that 1pg of *A. fumigatus* weights 10^{-12} g as its genome size is 2.9×10^7 bases. One base pair has a MW = 330×2 g/mol and 1 mol = 6.02×10^{23} molecules, we calculated that 1 pg of *A. fumigatus* DNA per μL of blood was equivalent to 1350 spores mL^{-1}. An identical method was used to determine spore counts in feed except the result being reported in spores/gr.

RESULTS

Two-step pan-fungal PCR assay: Samples from a dead cow which was fed with moldy corn silage were used in the assay to test for the presence of *A. fumigatus* DNA (Fig. 1 and 2). We also had access to a sample of aabomasal hemorrhage from a 3 years old gazelle. Although, abomasal hemorrhage was not the focus of this study, this sample was included to determine whether fungi could be detected in hemorrhagic abomasal wall.

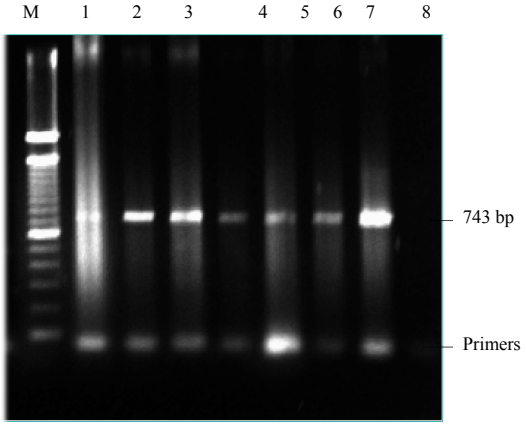

Fig. 1: First-round amplification in two-step PCR assay. Lane M is a ladder marker, lanes 1-7 are samples from the cow blood, feed, mesenteric lymph node, gut wall and gazelle hemorrhagic abomasum, *A. fumigatus* standard (1pg μL^{-1}), *A. fumigatus* standard (1 ng μL^{-1}). Lane 8 is a negative control (ddH2O). The target band is 743 bp.

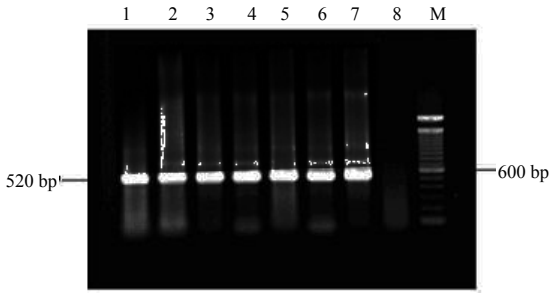

Fig. 2: Second-round (confirmatory) amplification in the two-step PCR assay. Nested PCR was completed using 1 μL of the first-round PCR product from lanes 1-8 (Fig. 2) as template. M is a MW ladder. The target band is 520bp

Results from first-round pan-fungal amplification of samples are shown in Fig. 1. Appearance of a 743 bp band represents detection of fungal DNA in the sample. All samples submitted including blood, feed, mesenteric lymph node, gut wall and gazelle abomasal wall (Lanes 2-8, Fig. 1) tested "positive" for fungi.

According to the positive control (1 pg μL^{-1} and 1 ng μL^{-1} *A. fumigatus* genomic DNA) in lanes 6, 7 and samples in lane 1 and 4 (Fig. 3), we detected about 200 fg μL^{-1} *A. fumigatus* ITS-DNA samples in the first PCR.

The second-round amplification (confirmatory) used nested primers (F2 and R2) and 1 μL^{-1} of the first-round

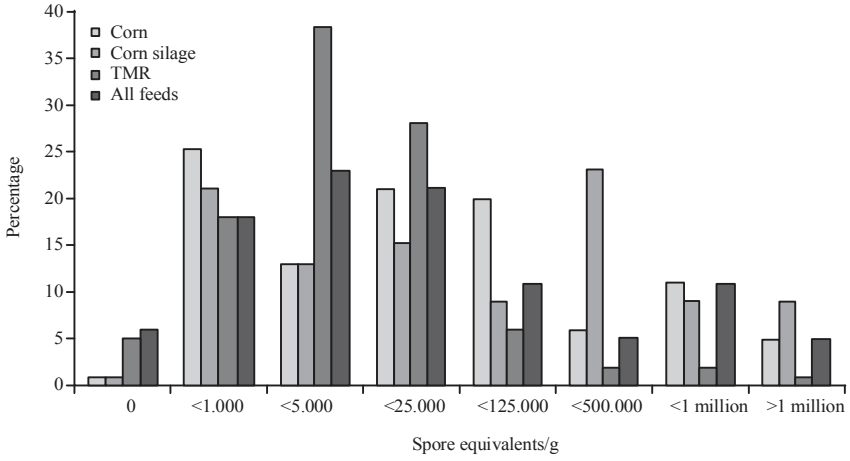

Fig. 3: The percent of *A. fumigatus* DNA contents form negative to >million spore equivalents/g in these corn, corn silage, TMR samples and all feed samples

Table 1: Detection of *A. fumigatus* DNA in samples of blood, tissues and feed recovered from seven cows. *A. fumigatus* levels were determined and reported as "---" (no detectable *A. fumigatus* DNA) and + to +++++ (low level to very high level). These correspond to *A. fumigatus* loads ranging from 0.2 - 1, 1 - 5, 5 - 20, 20 - 100 and >100 X 10^4*A. fumigatus* genomic units per g of tissue, per ml of blood or per ml of GI contents (+, ++, +++, ++++ and ++++, respectively). Spaces which are blank in Table 1 indicate that the sample was not available

Case identification	Feed	GI wall	GI contents	Mesenteric lymph node	Blood
MG #1		++	+++	+++++	++++
MG #2		+++	+++++	---	+++
HB #3		+++	+	---	+++
HB #4		+++	+++	+	++++
CC #1	+++				++
CC #2	++	+++++	+++++	++	
CC #3					+++++

PCR product from Fig. 3 as the template (Fig. 2). The *A. fumigatus*-specific nested primer set was designed to yield a 520 bp product. In addition, to the 520 bp PCR product, some non-specific bands, larger than the band of interest were also detected. These were assumed to be products carried-over fromthe pan-fungal PCR and the semi-nested PCR and were disregarded.

Real-time quantitative PCR: The same samples were used for the real-time quantitative PCR assays and included standards of *A. fumigatus* from 100 ng to 10 fg. There was no signal (amplification) in thenegative controls and the sensitivity appeared accurate down to 10 femtograms (10×10^{-15} g, 3 spores) in our standard curve. To generate the standard curve we performed a $\log_{10}$-transformationon a series of concentrations: 100 pg, 10 pg, 1 pg, 100 fg, 10 fg. With these C_T values, we obtained the graph and linear prediction equation. The R^2 for this example is 0.9953. This standard curve derivation was repeated every time samples were analyzed.

Because primer-dimer contaminated the total signal, we collected the fluorescence signal only above 83°C. Data analysis included the C_T value for standard DNA and we then used this to derive a formula from which we calculated the concentration of samples using Microsoft Excel.

The survey of *A. fumigatus* DNA contents in dairy feed across China: In the survey across China, 396 feed samples were collected over a year for analysis of *A. fumigatus* DNA content. From these, 95% tested positive with most ranging from 600-500,000 spores/g (Fig. 3). Ninety nine percent of the 30 corn silage samples tested positive. Of 82 TMR samples, only 5% tested negative and 1% of the 30 corn samples also tested negative.

A. fumigatus needs moisture and air for growth. Samples from deep within a well-preserved silage pit were not found to harbor *A. fumigatus* in significant quantities. Corn and grass silages accounted for all of the high testing samples (i.e., >1 million spore equivalents/g feed).

Detection of *A. fumigatus* DNA in GI tract, tissues and blood of ruminants using real-time quantitative PCR assay: Fungal burden is an *A. fumigatus* important concept in mycoses and, as result, we wanted information on *A. fumigatus* concentration. Table 1 outlines concentrations of *A. fumigatus* DNA in them.

Bloody GI contents were obtained from five cows and all tested positive for *A. fumigatus*. Portions of the GI lining associated with the clot were also tested and found

to harbor high concentrations of *A. fumigatus* (five of five cows tested). Mesenteric lymph nodes were recovered from five cows and of these, three tested positive. Perforation of the GI barrier (invasive aspergillosis) was evaluated by assaying blood and tissue concentrations. Six of six blood samples from cows contained *A. fumigatus*.

DISCUSSION

Diagnostic methods for fungal infection: Conventional diagnostic tools such as culture, histological and serological techniques are availablefor detection of invasive aspergillosis (Khan *et al*., 2010; Little and Warner, 2005; Reidarson *et al*., 1998). These methods have low specificity, poor sensitivity and reliability (Suarez *et al*., 2008; Biwas *et al*., 2008; Kappe and Rimek, 1999). Currently, available tests for fungal infections, usually take longer than a week, however, early diagnosis and rapid intervention are critical elements foran effective treatment formycotic infection. This has led to the development of culture-independent diagnostic tests.

PCR has been shown to be a highly-sensitive diagnostic tool for the detection of infectious fungi in diverse specimens. Our aim was to establish anew, highly-sensitive and -specific PCR assay for the rapid detectionof pathogenic *A. fumigatus* in blood, tissueand feed samples. In this study, we developed a two-step (nested; pan-fungal) and a quantitative (*A. fumigatus*-specific) PCR assay. In the two-step PCR, after the first-round PCR, we used an internal primerset for the subsequent second-round to increase sensitivity. Nested PCR assays improve both the detection sensitivity and specificity. In the first description of such a two-step PCR for the detection of *Aspergillus* spp. in serum samples of patients, Yamakami *et al*. (1996) reported that a PCR with two sets of 18S rRNA primers had considerably improved sensitivity compared to that of a PCR assay with a single set of primers. Nested PCR sensitivity can detect as little as 80 fg of a DNA/sample (Spiess *et al*., 2003). However, after nested PCR, we could not estimate the original template concentration.

Consequently, we used a real-time quantitative PCR method to measure the concentration of *A. fumigatus* DNA in blood and a wide variety of tissue and domestic feed samples. Our assay was designed to be specific for *A. fumigatus* which is the most common *Aspergillus* species that causes invasive disease. This system could potentially provide reliable results within 5 h of receiving a sample (including DNA extraction steps). Results obtained demonstrate the successful use of real-time PCR in this setting and suggest that this technique is more sensitive than regular PCR (as low as 10 fg).

Several critical points for *A. fumigatus* detection accuracy: Sample collection, transport as well as DNA extraction methods are critical parameters. In the study, we transported well-dry samples or wet samples with ice-bag overnight to the lab, we also optimized DNA extraction to remove the potential inhibitors, it is easy to co-purify inhibitors of the PCR that would generate inconsistent and unreliable results. Any question samples were repeated using 1:10 or 1:100 dilutions.

Application of methods to dairy industry: Early diagnosis and initiation of antifungal therapy are essential to reduce high associated rate of mortality, *A. fumigatus* accounts for approximately 90% of all cases of life threatening IA (Zafra *et al*., 2008; Chen *et al*., 2013). PCR based method has the potential to not only substantially increase the sensitivity of PCR but also to provide the means to monitor response to therapy by measuring fungal burden at any given point in time. From quantitative real-time assays, we determined total pathogen number, next step is to supply assistance in interpretation, whether a pathogen with particular concentration has the ability to establish an infection or not. In term of sampling, we recommended increasing the sampling size. And samples have been selected from farms where problems are being experienced and in many cases, highly-spoiled regions of feeds were selected for analysis. Hence, we believe that the infection rate of most feeds should be lower than that which is depicted. Another consideration is that feed varies in infection, depending upon location of the sample. *A. fumigatus* needs moisture and air for growth. Samples deep within a well-preserved silage pit, for example, typically do not harbor *A. fumigatus* in significant quantities. Good sampling strategy will help us to monitor *A. fumigatus* contamination and further to protect high risk group of dairy cows.

CONCLUSION

This study presents a rapid and sensitive detection of *A. fumigatus* in different types of samples and suggests that dairy cattle and other farm animals are facing a huge challenge from *A. fumigatus* contamination in domestic feed.

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