

# Quantification of airborne *Aspergillus fumigatus* spores in rabbit houses by real-time polymerase chain reaction

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**Abstract** To accurately quantify airborne *Aspergillus fumigatus* (*A. fumigatus*) spores in rabbit houses, the real-time polymerase chain reaction (real-time PCR) and culture-based counting method (CCM) were employed to determine the airborne *A. fumigatus* spore concentrations. The results showed that, of the three rabbit houses (A, B, and C), the average concentrations of airborne *A. fumigatus* spores determined by real-time PCR were  $3.0 \times 10^3$ ,  $3.3 \times 10^3$ , and  $1.5 \times 10^3$  spores/m<sup>3</sup> air, respectively, while those determined by CCM were  $2.5 \times 10^2$ ,  $2.8 \times 10^2$ , and  $1.1 \times 10^2$  colony-forming unit/m<sup>3</sup> air (CFU/m<sup>3</sup> air), respectively, i.e., the former concentration was 12–14 times higher than the latter one. Therefore, the conventional CCM underestimated the concentrations of airborne fungal spores, and it is insufficient to determine the microbial aerosol concentration and evaluate the health risk only using CCM.

**Keywords** Airborne *Aspergillus fumigatus* spore · Rabbit houses · Real-time polymerase chain reaction · Culture-based counting method · Health risk

## 1 Introduction

*Aspergillus fumigatus* (*A. fumigatus*), the principal aetiological agent of invasive aspergillosis, belongs to the genus *Aspergillus* section *Fumigati* (Tomee and Kauffman 2000; Pini et al. 2004). Pulmonary infection may easily occur in susceptible persons when they inhale *A. fumigatus* spores (Cruz-Perez et al. 2001). *A. fumigatus* is the second important pathogenic fungus following *Candida albicans* and its toxins might directly cause damage to human body or decrease immunity of human body (Wang and Feng 2007). *A. fumigatus* is also a great hazard to animals, for example, its toxins might cause equine leucoencephalomalacia in horses, and respiratory diseases in rabbits and quails (Zhang et al. 1997). Studies have demonstrated that if chicks are exposed to an environment where the *A. fumigatus* spore concentration reaches  $10^9$  CFU/m<sup>3</sup> air, acute respiratory symptoms might occur in several weeks; and if *A. fumigatus* spore concentration reaches  $10^{10}$  CFU/m<sup>3</sup> air or higher, acute respiratory symptoms might occur in only one day (Richard et al. 1981; Malmberg and Rask-Andersen 1988).

The conventional method to determine microbial aerosol concentration is the culture-based counting method (CCM). However, it was reported that only

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0.3–10% of microorganisms may be cultivated for counting; therefore, CCM may easily underestimate microbial aerosol concentration. But uncultivated microorganisms and their metabolites may also cause adverse reactions or infections of animals and human beings (Zhou et al. 2000). With the development of molecular biological technology, the introduction of real-time polymerase chain reaction (real-time PCR) has offered the ability of simultaneous detection and quantification of DNA of a specific microbe in one reaction. And its main advantages are its quantitative property, high sensitivity, high specificity, and no post-PCR steps (O'Sullivan et al. 2003; Liu et al. 2009).

Therefore, in this study, the airborne *A. fumigatus* spore concentrations in rabbit houses were determined by real-time PCR and CCM, and meanwhile the results were compared.

## 2 Materials and methods

### 2.1 Standard strain and genomic DNA extraction

The standard strain of *A. fumigatus* (ATCC 10894) was obtained from the China General Microbiology Culture Center (CGMCC). *A. fumigatus* was cultivated on the potato dextrose agar culture medium (PDA, Beijing Road and Bridge Technology Company) at 28°C (Zhang et al. 1997; Wang and Feng 2007; Liu et al. 2009). The genomic DNAs were isolated from the pure culture of the fungal strain by using the procedure described previously by Liu et al. (2009) and the instruction of the Kit (AxyPrep Multisource Genomic DNA Miniprep Kit, AXYGEN Company, USA).

### 2.2 Standard curve

Standard curve based on threshold cycles at which the fluorescence signal exceeds the background during the exponential phase of amplification was constructed by using tenfold dilution series of *A. fumigatus* DNA ( $10^2$ – $10^7$  genomic DNAs/ $\mu$ L). A 3  $\mu$ L aliquot of each dilution, in three replicate assays, was used in real-time PCRs with the prime and probe (Mto1F-5'-tttctccacc-caggaacgtt-3'; Mto1R-5'-cgaatccggagaggtgatacc-3'; Taqman's probe sequence: 5'-FAM-cagttgtgatgacgacagccagc-TAMRA-3' (Liu et al. 2009). The primer and probe were synthesized by Shanghai GeneCore Biotechnologies Co., Ltd.. After amplification, the

standard curve was automatically generated by the software SDS V 1.9.1 of the PCR Thermal Cycler (ABI 9700 type PCR Thermal Cycler, USA).

### 2.3 Sampling site, collection, and treatment of samples and real-time PCR

A rabbit-breeding farm in Shandong Qingdao Kangda Food Co., Ltd., 15 km away from the downtown, was selected for the detection of *A. fumigatus* spores. The rabbit houses were 60 m  $\times$  6 m  $\times$  3 m in volume; 2,000 healthy adult rabbits were bred with feed particles in each house, under positive-pressure ventilation, at temperature 26°C, and relative humidity 78% controlled by the air-conditioner.

Sampling was simultaneously carried out in three rabbit houses (A, B, and C) randomly selected from the rabbit-breeding farm. Airborne particles were collected into 50 mL sterile distilled water by all glass impinger-30 (AGI-30), which is useful for the sampling of cultivable or uncultivable airborne particles (Brachmann et al. 1964). The air flow rate was 12.5 L/min, and the sampling time was 30 min (Lin et al. 1997). The sampling flow rate and time were identical for all the three samplers. Six samples were collected from each rabbit house (Pini et al. 2004; Zeng et al. 2004, 2006).

After sampling, colony counting was performed by spreading 25 mL of each sample onto 25 PDA plates. The 25 plates were incubated at 28°C for 5–7 days before the CFU (colony-forming unit, CFU/m<sup>3</sup> air) was determined (Iwen et al. 1994; Zhang et al. 1997; Wang and Feng 2007). Another 25 mL of each sample was used for DNA extraction according to the method described by Wu et al. (2002). The extracted DNAs were analysed by real-time PCR following the conditions described by Liu et al. (2009). Through real-time PCR, the quantity of *A. fumigatus* DNA used as the original template in each reaction was calculated from the standard curve from which the concentration of *A. fumigatus* spores in the sample was deduced. Each air sample was repeated three times in the real-time PCR analysis.

## 3 Results

### 3.1 Standard curve

It can be seen from the generated standard curve in this study that the correlation coefficient, the slope, and the

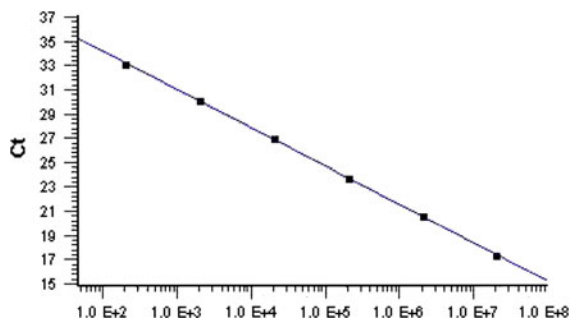


**Fig. 1** Standard curve generated from the amplification of tenfold dilutions from target genomic DNA from ATCC 10894. Horizontal axis denotes starting numbers of genomic DNAs; Vertical axis denotes cycle threshold (Ct)

intercept were 0.99985254,  $-3.1564424$ , and  $40.494341$ , respectively. The standard curve equation was  $y = -3.1564424x + 40.494341$ , and the  $r^2$  value was up to 0.99985254 (See Fig. 1).

### 3.2 Concentration of airborne *A. fumigatus* spores

Eighteen samples of three rabbit houses were detected by real-time PCR, and the results revealed that the average concentrations of airborne *A. fumigatus* spores in the three rabbit houses (A, B, and C) were  $3.0 \times 10^3$ ,  $3.3 \times 10^3$ , and  $1.5 \times 10^3$  spores/ $\text{m}^3$  air, respectively. Meanwhile, the 18 samples were also analysed through the cultivation on PDA medium. The results showed that the average concentrations of airborne *A. fumigatus* spores in the three rabbit houses (A, B, and C) were  $2.5 \times 10^2$ ,  $2.8 \times 10^2$ , and  $1.1 \times 10^2$  CFU/ $\text{m}^3$  air, respectively (See Table 1).

## 4 Discussion

The real-time PCR assay is based on measuring the fluorescence released during primer extension as the

5'-nuclease activity of Taq DNA polymerase cleaves a dual-labelled fluorescent hybridization probe, designed to bind inside the amplified region. The real-time PCR has the ability of simultaneous detection and quantification of DNA of a specific microbe in one reaction. Its main advantages are quantitative property, high sensitivity, high specificity, and no post-PCR steps (Jiang et al. 2008).

The specificity and detection sensitivity of the real-time PCR system used in this experiment have been analysed by Liu et al. (2009); the results showed that the presence of other fungal background did not affect the detection of *A. fumigatus* DNA, with detection sensitivity up to  $1.08 \times 10^{-5}$  ng/ $\mu\text{L}$  (Liu et al. 2009). Thus, the real-time PCR system used in this study could potentially detect *A. fumigatus* spore in a reaction.

Eighteen aerosol samples were all analysed by the real-time PCR and CCM. Among all the 18 air samples, the highest average concentrations of *A. fumigatus* spores were observed in the rabbit house B ( $3.3 \times 10^3$  spores/ $\text{m}^3$  air;  $2.8 \times 10^3$  CFU/ $\text{m}^3$  air). Compared with the cultivation result, the real-time PCR quantification was about 12–14 times higher than CCM, which was consistent with the results in the studies conducted by Zeng et al. (2004, 2006). This difference can be explained by the presence of unviable spores and hyphal fragments in the aerosols that originated from old fungal colonies and multiple-spore aggregates that form single colonies.

However, viable but non-cultivable microorganisms and dead cells that cannot be detected by CCM might also produce hazard to animals and human beings (Sehlesinger 1977; McDougald et al. 1998). Moreover, high concentration of dead cells and their metabolites may cause allergic symptoms; therefore, they cannot be ignored in assessing the health risk of environments (Shahamat et al. 1997). In the real-time PCR detection, all the collected bio-particles are analysed regardless of their cultivability. Thus, the real-time PCR gave estimates that better reflect the true concentration of airborne *A. fumigatus* spores. Contaminants, spore viability, growth medium, and the abundance of other fast-growing fungi could all have affected the detection of *A. fumigatus* colonies (Crook and Sherwood-Higham 1997; Wu et al. 2000); therefore, it is insufficient to determine the microbial aerosol concentration and evaluate the health risk only using CCM.

**Table 1** Average concentrations of airborne *A. fumigatus* spores

| Rabbit houses (N) | Real-time PCR (spores/ $\text{m}^3$ air) | CCM (CFU/ $\text{m}^3$ air) |
|-------------------|------------------------------------------|-----------------------------|
| A (6)             | $3.0 \times 10^3$                        | $2.5 \times 10^2$           |
| B (6)             | $3.3 \times 10^3$                        | $2.8 \times 10^2$           |
| C (6)             | $1.5 \times 10^3$                        | $1.1 \times 10^2$           |

N the number of air samples

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