Development and Validation of a Multiplex Real-Time PCR Assay for Simultaneously Detection of *Aspergillus*, *Cryptococcus neoformans*, and *Pneumocystis jirovecii*

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INTRODUCTION

- Approximately 6.5 million new cases of invasive fungal infections (IFI) and 3.8 million deaths are reported annually, affecting over 1 billion people worldwide.
- ***Aspergillus, Cryptococcus, and Pneumocystis* are the common pathogens leading to IFI.**

AIM

- **In this study, a multiple real-time polymerase chain reaction (PCR) assay was developed to simultaneously detect and identify three of the most frequent pathogens in sputum and bronchoalveolar lavage fluid (BALF) samples (Figure 1), including *Aspergillus, Cryptococcus neoformans, and Pneumocystis jirovecii*, and to evaluate its performance on clinical samples.**

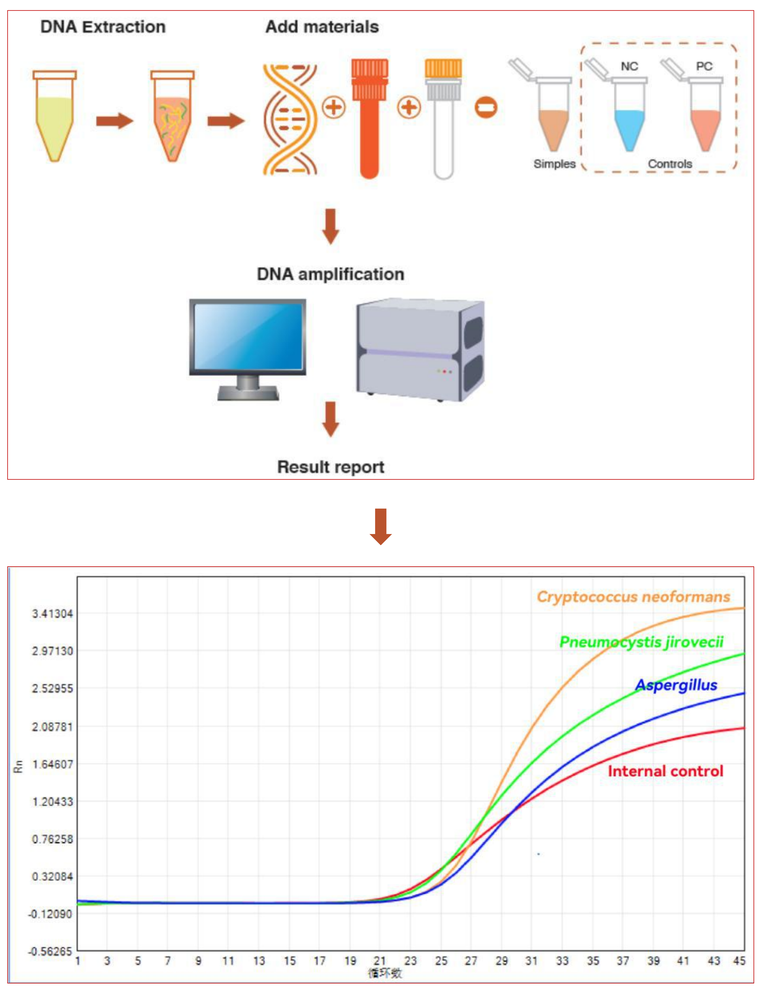

Figure 1 Detection Procedure of the Multiplex Real-time PCR assay.

METHOD

- Sputum samples (n=244) and BALF samples (n=299) from patients with suspected IFI were collected from three centres. **The diagnostic sensitivity and specificity of the multiplex real-time PCR assay were analysed.**
- DNA sequencing was used as the reference method, and the performance of the multiplex real-time assay was evaluated by determining the **positive percent agreement (PPA), negative percent agreement (NPA), and overall coincidence rate**.
- **The reproducibility, limit of detection (LoD), and interference experiment of the multiplex real-time PCR assay were also evaluated**.

RESULTS

➢From the clinical evaluation results, the sensitivity and specificity of the multiplex real-time PCR assay were **greater than 90%** for *Aspergillus, Cryptococcus neoformans, and Pneumocystis jirovecii* (Table 1).

➢The consistency of the multiplex real-time PCR assay and DNA sequencing was almost perfect: the overall coincidence rate of the two methods **was close to 100%** (Table 2).

➢The percent coefficient variations (CVs%) of reproducibility **was less than 5%,** meeting requirements, and the LoD of the kit for each of the three species **less than or equal to 1000 copies/mL** (Table 3).

➢**No cross-reactivity** was detected for any bacteria or *fungi*.

Table 1 Clinical performance of the multiplex real-time PCR assay

Sample type	Target Organism	Sensitivity (%)	Specificity (%)
Sputum	*Aspergillus*	**94.94 (75/79)**	**97.59 (81/83)**
	Cryptococcus neoformans	90.91 (20/22)	96.39 (80/83)
	Pneumocystis jirovecii	92.50 (74/80)	96.39 (80/83)
BALF	*Aspergillus*	97.06 (132/136)	100.00 (58/58)
	Cryptococcus neoformans	92.00 (46/50)	98.28 (57/58)
	Pneumocystis jirovecii	94.55 (52/55)	98.28 (57/58)

Table 2 Comparison between the multiplex real-time PCR assay and DNA sequencing results in sputum and BALF

Sample type	Target Organism	Positive percent agreement (%)	Negative percent agreement (%)	Overall percent agreement (%)
Sputum	*Aspergillus*	100.00 (79/79)	100.00 (83/83)	100.00 (162/162)
	Cryptococcus neoformans	95.45 (21/22)	100.00 (83/83)	99.05 (104/105)
	Pneumocystis jirovecii	97.50 (78/80)	100.00 (83/83)	98.77 (161/163)
BALF	*Aspergillus*	100.00 (136/136)	100.00 (58/58)	100.00 (194/194)
	Cryptococcus neoformans	98.00 (49/50)	100.00 (58/58)	99.07 (107/108)
	Pneumocystis jirovecii	100.00 (55/55)	100.00 (58/58)	100.00 (113/113)

Table 3 Verification of the LoD of the multiplex real-time PCR assay

Sample type	Target Organism	LoD (1000 copies/mL)
Sputum	*Aspergillus*	**100% (20/20)**
	Cryptococcus neoformans	95% (19/20)
	Pneumocystis jirovecii	100% (20/20)
BALF	*Aspergillus*	100% (20/20)
	Cryptococcus neoformans	100% (20/20)
	Pneumocystis jirovecii	100% (20/20)

CONCLUSIONS

The multiplex real-time PCR assay was established with high sensitivity and specificity for rapid and simultaneous **detection on three common fungal pathogens in respiratory tract samples.**