Infection and Drug Resistance

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ORIGINAL RESEARCH

Evaluation of the Performance of a Multiplex Real-Time PCR Assay for the Identification of *Aspergillus, Cryptococcus neoformans*, and *Pneumocystis jirovecii* Simultaneously from Sputum in Multicenter

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Purpose: To evaluate the performance of a multiplex real-time polymerase chain reaction (PCR) assay for the simultaneous identification of *Aspergillus, Cryptococcus neoformans*, and *Pneumocystis jirovecii* from sputum.

Patients and Methods: Sputum samples (n=537) from patients with suspected invasive fungal infection (IFI) were collected from four centers; they were tested by both multiplex real-time PCR assay and DNA sequencing. DNA sequencing was considered as the reference method, and the performance of the multiplex real-time assay was evaluated by determining the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). The interference experiment, repeatability, reproducibility, and stability of the multiplex real-time PCR assay were also evaluated.

Results: The detection performance of the multiplex real-time assay, compared with that of DNA sequencing, for the three pathogens was as follows: sensitivity, specificity, PPV, and NPV for *Aspergillus, Cryptococcus neoformans*, and *Pneumocystis jirovecii* were 99.40%, 98.64%, 97.09%, and 99.73%; 100%, 99.59%, 96.36%, and 100.00%; and 99.28%, 98.50%, 95.80%, and 99.75%, respectively. The consistency of the two methods was almost perfect: the kappa value was between 0.97 and 0.98. The minimum detection limit of the multiplex real-time assay for each of the three pathogens was 1250 copies/mL. Interference experiment showed that blood and normally used antifungal drugs had no effect on the results. No cross-reactivity was detected for any bacteria or fungi. In 40 patients, mixed infections by *Aspergillus* and/or *Cryptococcus neoformans* and/or *Pneumocystis jirovecii* were detected by the multiplex real-time assay. Among these patients, those with acquired immune deficiency syndrome (AIDS) ranked first, with *Aspergillus* and *Pneumocystis* mixed infection accounting for 75%.

Conclusion: The multiplex real-time PCR assay is fast, sensitive, and specific and has good clinical application prospects.

Keywords: *Aspergillus*, *Cryptococcus neoformans*, *Pneumocystis jirovecii*, multiplex real-time PCR, sequencing, invasive fungal infection

Introduction

The prevalence and mortality of invasive fungal infections (IFI) have shown a significant upward trend, *Candida, Aspergillus, Cryptococcus*, and *Pneumocystis* are the common pathogens leading to IFI.[1,2] The incidence of IFI is

Received: 18 June 2022
Accepted: 28 September 2022
Published: 18 October 2022

increasing, and mixed infections by different pathogens make it more difficult to achieve an accurate diagnosis. The true burden of IFI is probably underestimated because of the absence of reliable diagnostics methods.[3]

The conventional methods for diagnosing *Aspergillus* and *Cryptococcus* are mainly based on isolation on a designated medium, which is time-consuming; *Pneumocystis* diagnosis depends on Grocott's methenamine silver stain microscopy, and the sensitivity and specificity of such methods are limited.[4,5] The diagnostic process is complex, and therefore, clinical, radiological, and microbiological findings should be considered comprehensively.[6]

Genome sequencing has led to improved understanding of the epidemiology of less common fungal infections. However, sequencing requires special equipment and is expensive.[5,7] Polymerase chain reaction (PCR)-based technologies have been widely used to detect a number of human pathogens. Real-time PCR-based assays are designed to target a single organism at a time. When screening multiple targets, high-throughput multiplex assays are critically needed, reducing the time and cost.[8] Recently, some reports have described the utility of multiplex real-time PCR protocols to increase the sensitivity and specificity of diagnosis of special fungi.[3,9–11] We aimed to use this method in the current study to simultaneously detect three common pathogens, *Aspergillus, Cryptococcus neoformans*, and *Pneumocystis jirovecii* in sputum samples.

Materials and Methods

Samples

A total of 541 sputum samples were collected from patients with suspected *Aspergillus, Cryptococcus neoformans*, and *Pneumocystis* infection from four central hospitals from September 2020 to July 2021: Peking Union Medical College Hospital of the Chinese Academy of Medical Sciences, Beijing Ditan Hospital Affiliated to Capital Medical University, the First Affiliated Hospital of Bengbu Medical College, and Chongqing Public Health Medical Treatment Center, which are distributed in North China, East China and Southwest China. As the exclusion criterion, samples repeatedly isolated from the same patient were excluded. This study was approved by the Ethics Committee of Peking Union Medical College Hospital (ethical approval number: KS 2019560). The requirement for informed consent was waived because the data did not compromise patient safety and privacy.

DNA Extraction

Sample pretreatment: The sputum sample was transferred to a 50 mL screw capped test tube, and 1–2 times volume of 4% NaOH digestive solution was added to the sputum tube. The tube was tightened, oscillated for 1 min, and then stored at room temperature for 15–20 min to fully liquefy the sputum. The DNA of positive and negative controls and samples was extracted simultaneously according to the manufacturer's instructions (Nucleic acid extraction kit, Beijing Zhuo Cheng Hui Sheng Biotechnology Co., Ltd., Beijing, China). All operations were performed in a biosafety cabinet.

Multiplex Real-Time PCR

Real-time PCR for *Aspergillus, Cryptococcus neoformans*, and *Pneumocystis* DNA was performed using three multiplex real-time PCR detection kits (PCR fluorescence probe method, Beijing Zhuo Cheng Hui Sheng Biotechnology Co., Ltd., Beijing, China). The methodology involved species-specific molecular primers and beacon probes targeting the 18S rDNA of *Aspergillus*, the internal transcribed spacer (ITS) regions of *Cryptococcus neoformans*, and the mitochondrial large subunit RNA (mtLSUrRNA) of *Pneumocystis*. An internal control targeting the human housekeeping gene Ribonuclease P (RNase P) was included in each assay.

The test protocol was as follows: PCR was performed using a reaction mixture containing 35 μL of nucleic acid amplification reaction solution, 5 μL of primer-probe mixture, and 10 μL of extracted DNA to a final volume of 50 μL. The reaction conditions included 1 cycle of pre-incubation at 50 °C for 2 min, denaturation at 95 °C for 5 min, followed by 40 PCR cycles constituting denaturation at 95 °C for 15s, annealing/extension and signal acquisition at 60 °C for 45s, followed by a 1 min instrument hold at 12 °C. Negative and positive controls were included in each assay, and positive controls with all targeted sequences were provided in the kit. The final results were analyzed using the ABI 7500 fluorescence quantitative PCR software (Thermo Fisher Scientific, Massachusetts, USA).

Positive judgment value: the FAM channel cycle threshold (Ct) value of ≤33.7 indicated positivity for *Aspergillus*; the VIC channel Ct value of ≤36 indicated positivity for *Cryptococcus neoformans*; the CY5 channel Ct value of ≤36 indicated positivity for *Pneumocystis*.

Species Identification by DNA Sequencing

Definitive identification was performed by sequencing the ITS region from the rDNA with the primer sequences and PCR conditions described previously.[12] The PCR products were purified, and sequencing was performed using an ABI 3730 DNA analyzer (Thermo Fisher Scientific, Massachusetts, USA). The sequences were compared using the BLAST algorithm in the National Center for Biotechnology Information GenBank database (http://www.ncbi.nlm.nih.gov). Species identification was based on the similarity value of equal to or greater than 99.0% for the ITS region.[12]

Statistical Analysis

DNA sequencing was used as the reference method, and the performance of the real-time multiplex assay was evaluated by determining its sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) using SPSS Statistics 26.0 software. McNemar's test was used to analyze the sensitivity and specificity of the two diagnostic tests. Agreement between the two tests was determined using Cohen's kappa (κ) with 95% confidence intervals (CI). The degree of statistical significance was set at $P < 0.05$.

Results

Medical Record Review

A total of 541 sputum samples were included and tested. Finally, 537 valid data points were obtained after removing the samples that could not be sequenced. The demographic information showed that there were 370 men (68.90%) and 167 women (31.10%); the average age was 57 years, with minimum age of 1 month and 14 days and maximum of 94 years.

The distribution of the clinical diagnosis of patients was as follows: 142 (26.44%) patients with acquired immune deficiency syndrome (AIDS); 105 (19.55%) with other lung diseases (pulmonary cystic fibrosis, bronchiectasis, chronic obstructive pulmonary disease, lung shadow, etc.); 102 (18.99%) with pulmonary infection (pulmonary bacterial or fungal infection, pulmonary tuberculosis, chronic obstructive pneumonia, etc.); 44 (8.19%) with lung cancer and 56 (10.43%) with other cancers; 16 (2.98%), 11 (2.05%), 18 (3.35%), 12 (2.23%), and 31 (5.77%) with immune diseases, hematological malignancies, solid organ transplantation, basic diseases such as cardio cerebrovascular disease, diabetes, and other kind of infection diseases, and other immunocompromised patients (liver failure, renal insufficiency, etc.), respectively.

Multiplex Real-Time Assay Performance

The minimum detection limit of the kit for each of the three species was 1250 copies/mL. DNA sequencing was used as the gold standard; the performance of the multiplex real-time assay for detecting *Aspergillus*, *Cryptococcus neoformans*, and *Pneumocystis* was analyzed separately.

Detection of *Aspergillus* Spp

The distribution of *Aspergillus* species (referred to as DNA sequencing) was as follows: 78 (46.71%) *Aspergillus fumigatus*, 61 (36.53%) *Aspergillus flavus*, 11 (6.59%) *Aspergillus niger*, 7 (4.19%) *Aspergillus oryzae*, 4 (2.40%) *Aspergillus variegatus*, 3 (1.80%) *Aspergillus terrestris*, 2 (1.20%) *Aspergillus nidus*, and 1 (0.60%) *Aspergillus ustus*.

As shown in Table 1, the detection performance of the multiplex real-time assay for *Aspergillus*, compared with that of DNA sequencing, was as follows: the sensitivity was 99.40%%; specificity 98.64%; PPV 167/172 = 97.09% (95% CI: 93. 35–99.05%); NPV 364/365 = 99.73% (95% CI: 98.48–99.99%); and total coincidence rate (167 + 364) /537 = 98.88% (95% CI: 97.58–99.59%).

The consistency of the two methods was good, kappa = 0.97≥0.75, $P< 0.001$; the pair $\chi 2$ McNemer test result showed that the difference between the two methods was statistically insignificant, $P = 0.22 > 0.05$, the overall coincidence rate of the two methods was the same.

Table 1 Multiplex Real-Time Assay versus DNA Sequencing for Detecting *Aspergillus*

Prediction Method \ Gold Method		DNA Sequencing		Total	
		Condition Positive	Condition Negative		
Multiplex real-time assay	Prediction positive	Ture positive=167	False positive=5	172	PPV =167/172 =97.09%
	Prediction negative	False negative=1	True negative=364	365	NPV =364/365 =99.73%
Total		168	369	537	
		Sensitivity =167/168 =99.40%	Specificity =364/369 =98.64%		

Abbreviations: PPV, positive predictive value; NPV, negative predictive value.

Detection of *Cryptococcus neoformans*

As shown in Table 2, the detection performance of the multiplex real-time assay for *Cryptococcus neoformans*, compared with that of DNA sequencing, was as follows: the sensitivity was 100%; specificity 99.59%%; PPV 53/55 = 96.36% (95% CI: 87.47–99.56%); NPV 482/482=100.00% (95% CI: 99.24–100.00%); and total coincidence rate (53 + 482) /537 = 99.63% (95% CI: 98.66–99.95%).

The consistency of the two methods was good, kappa = 0.98≥0.75, $P < 0.001$; the pair $\chi 2$ McNemer test result showed that the difference between the two methods was statistically insignificant, $P = 0.50 > 0.05$, the overall coincidence rate of the two methods was the same.

Detection of *Pneumocystis*

As shown in Table 3, the detection performance of the multiplex real-time assay for *Pneumocystis*, compared with that of DNA sequencing, was as follows: the sensitivity was 99.28%%; specificity 98.50%%; PPV 137/143 = 95.80% (95% CI: 91.09–98.44%); NPV 393/394=99.75% (95% CI: 98.59–99.99%); and total coincidence rate (137 + 393) /537 = 98.70% (95% CI: 97.33–99.47%).

Table 2 Multiplex Real-Time Assay versus DNA Sequencing for Detecting *Cryptococcus Neoformans*

Prediction Method \ Gold Method		DNA Sequencing		Total	
		Condition Positive	Condition Negative		
Multiplex real-time assay	Prediction positive	Ture positive=53	False positive=2	55	PPV =53/55 =96.36%
	Prediction negative	False negative=0	True negative=482	482	NPV =482/482 =100%
Total		53	484	537	
		Sensitivity =53/53 =100%	Specificity =482/484 =99.59%		

Abbreviations: PPV, positive predictive value; NPV, negative predictive value.

Table 3 Multiplex Real-Time Assay versus DNA Sequencing for Detecting *Pneumocystis*

Prediction Method \ Gold Method		DNA Sequencing		Total	
		Condition Positive	Condition Negative		
Multiplex real-time assay	Prediction positive	Ture positive=137	False positive=6	143	PPV =137/143 =95.80%
	Prediction negative	False negative=1	True negative=393	394	NPV =393/394 =99.75%
Total		138	399	537	
		Sensitivity =137/138 =99.28%	Specificity =393/399 =98.50%		

Abbreviations: PPV, positive predictive value; NPV, negative predictive value.

The consistency of the two methods was good, kappa = 0.97≥0.75, $P < 0.001$; the pair $\chi 2$ McNemer test result showed that the difference between the two methods was statistically insignificant, $P = 0.13 > 0.05$, the overall coincidence rate of the two methods was the same.

Two or Three Pathogens Detected Simultaneously by the Multiplex Real-Time Assay

Two or three pathogens can be simultaneously detected using multiplex real-time assays. The PCR amplification curves of simultaneous positivity for two or three pathogens are shown in Figure 1. The types of pathogen combinations and inconsistent results with DNA sequencing are shown in Table 4. Among the patients with mixed infection, those with AIDS ranked first at 40% (16/40), followed by those with lung infection and other lung diseases at 20% (8/40).

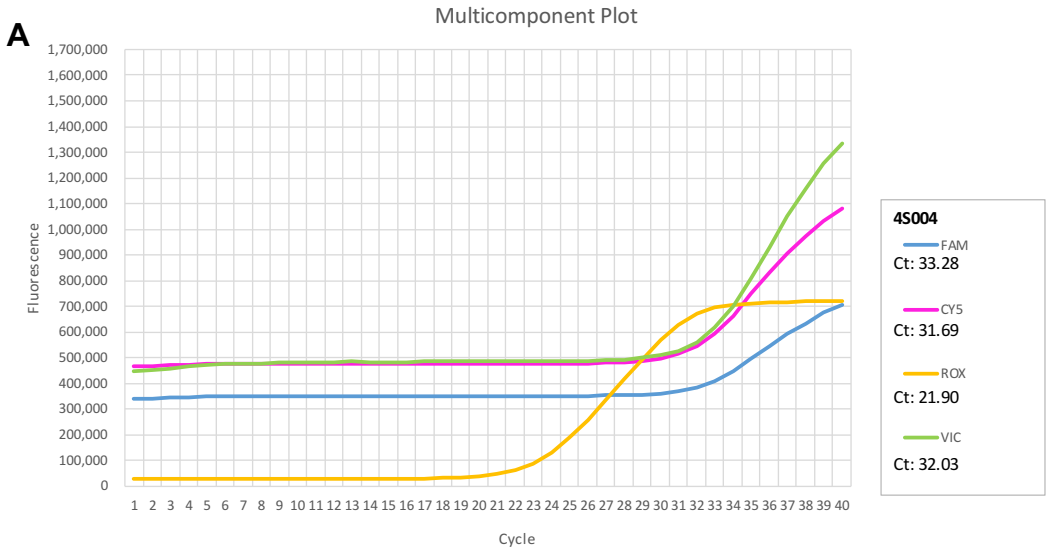

Figure 1 Continued.

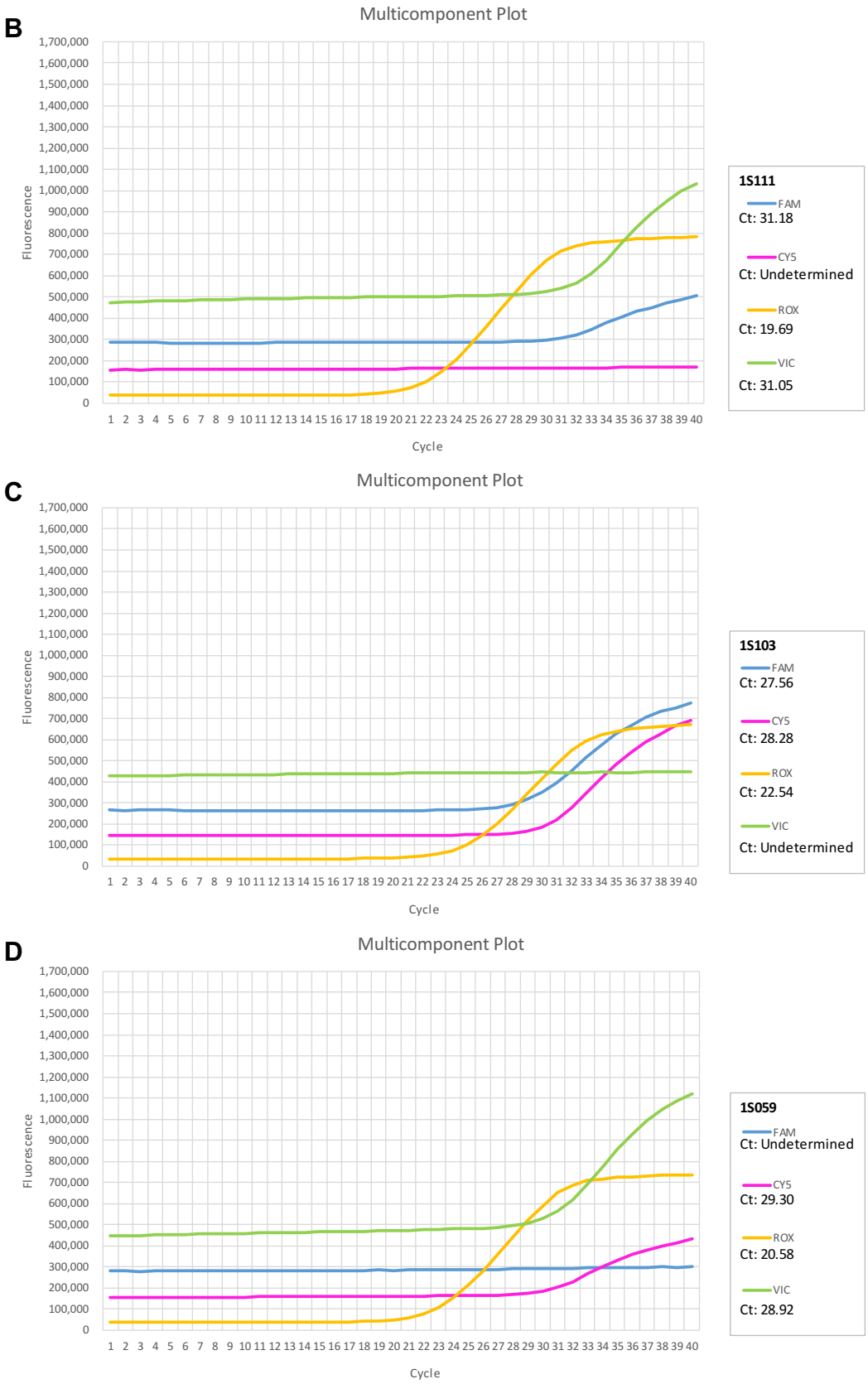

Figure 1 PCR amplification curves of simultaneous positive for two or three pathogens. (**A**) *Aspergillus*, *Cryptococcus neoformans*, and *Pneumocystis jirovecii* were all positive. (**B**) *Aspergillus*, and *Cryptococcus neoformans* were both positive, *Pneumocystis jirovecii* was negative. (**C**) *Aspergillus* and *Pneumocystis jirovecii* were both positive, *Cryptococcus neoformans* was negative. (**D**) *Cryptococcus neoformans* and *Pneumocystis jirovecii* were both positive, *Aspergillus* was negative. PCR channel: *Pneumocystis jirovecii*: CY5; *Aspergillus*: FAM; Internal control: ROX; *Cryptococcus neoformans*: VIC.

Table 4 The Pathogen Combinations with More Than Two Positive Results by Multiplex Real-Time Assay and DNA Sequencing

Multiplex Real-Time Assay		DNA Sequencing
Pathogens detected simultaneously	N	Different results from multiplex real-time assay
Aspergillus, Cryptococcus neoformans, and *Pneumocystis*	3	One sample is positive for *Aspergillus* and *Cryptococcus neoformans* but negative for *Pneumocystis*
Aspergillus and *Cryptococcus neoformans*	5	One sample is negative for both *Aspergillus* and *Cryptococcus neoformans*
Aspergillus and *Pneumocystis*	30	One sample is positive for *Aspergillus* but negative for *Pneumocystis*Two samples are positive for *Pneumocystis* but negative for *Aspergillus*
Cryptococcus neoformans and *Pneumocystis*	2	0

Abbreviation: N, number of specimens.

Discussion

Most patients with IFI have no characteristic clinical symptoms and are easily misdiagnosed as having primary bacterial and/or viral infections, which makes clinical diagnosis difficult. Immune deficiency, hematological malignancy, transplantation, treatment with immunosuppressive drugs or corticosteroids, admission to the intensive care unit ward, structural pulmonary disease and/or complicated influenza, and poorly controlled diabetes are high clinical risk factors for IFI.[6] In this study, pulmonary diseases and AIDS and other immunocompromised diseases were the top two clinical diagnoses. AIDS was also the most common diagnosis for patients with two or three positive results for the target pathogens.

In this study, the results of fungal culture and Grocott's methenamine silver stain for all patients were analyzed retrospectively; both multiplex real-time assay and DNA sequencing showed positive results according to the evaluation criteria, and the positive rates of culture for *Aspergillus* and *Cryptococcus neoformans* were 44.91% (75/167) and 5.66% (3/53), respectively. The positive rate of Grocott's methenamine silver stain for *Pneumocystis* was 28.47% (39/137). The low sensitivity of culture may be related to factors such as taking part of sample for inoculation, culture conditions, difficulty in isolating after antifungal treatment, and the inexperience of technicians,[13] which may lead to misdiagnosis or missed diagnosis.[5]

In this multiplex real-time PCR assay for the detection of *Aspergillus, Cryptococcus neoformans*, and *Pneumocystis*, the minimum detection limit for each species was 1250 copies/mL. When comparing with DNA sequencing, the sensitivity, specificity, PPV, NPV, and total coincidence rate were between 95.80% and 100%. The consistency between the two methods was almost perfect, and the kappa value was between 0.97 and 0.98. The common bacteria, fungi, commonly used antifungal drugs, and blood in the sample had no effect on the PCR results. The assay also exhibited good repeatability, reproducibility, and stability, indicating that it had good clinical application prospects.

In this study, eight *Aspergillus* species were detected by multiplex real-time assay; the top three species were *Aspergillus fumigatus, Aspergillus flavus*, and *Aspergillus niger*, which was the same as that in a previous study.[14] If the result of the multiplex real-time assay was positive but the DNA sequencing result was negative, it was defined as "B"; if the result of the multiplex real-time assay was negative but the DNA sequencing result was positive, it was defined as "C". One B (n=5) sample of *Aspergillus* was cultured as *Aspergillus terreus*, and the only one C (n=1) sample of *Aspergillus* was cultured as *Aspergillus spp*. Both multiplex real-time PCR assay and DNA sequencing indicated 393 samples as *Pneumocystis jirovecii* negative, of which 9 cases were clinically diagnosed as *Pneumocystis* pneumonia with negative results by Grocott's methenamine silver stain. This may be related to the disease stage or to the collection and preservation of samples[14] or to receiving antifungal prophylaxis that can significantly reduce the PPV of PCR. Therefore, if IFI is considered, multiple methods are suggested for screening, and comprehensive judgment should be based on clinical symptoms and other results.[15]

This study however has limitations. Regarding the positive results of *Aspergillus* and *Pneumocystis* by multiplex real-time PCR assay, colonization or infection still required to be distinguished. This is important for balancing the potential risk of long-term administration of drugs in patients who might have a risk of pneumonia, avoiding over-treatment of patients without IFI, or missing the target in the case of resistance.[2,16,17] Further well-designed methodologies considering clinical diagnosis as the gold standard are needed to evaluate the clinical utility of this assay.

Conclusion

Early diagnosis of IFI, including accurate identification of the causative fungus, is critical for appropriate patient management and improving outcomes, especially for central nervous system infections with higher mortality.[11] The multiplex real-time PCR assay in this study had good sensitivity, specificity, PPV, and NPV and identified multiple pathogens simultaneously. Thus, it has good clinical application prospects that may potentially lead to early diagnosis and treatment.

Abbreviations

PCR, polymerase chain reaction; PPV, positive predictive value; NPV, negative predictive value; AIDS, acquired immune deficiency syndrome; CI, confidence interval; Ct, cycle threshold; IFI, invasive fungal infection; ITS, internal transcribed spacer.

Data Sharing Statement

The data used and/or analyzed in this study are available from the corresponding author upon reasonable request.

Ethical Approval

This study involved the anonymous use of redundant and abandoned patient sputum after laboratory testing, with no contact with the patient, and no patient identification was performed during data collection. This research did not affect patients' health and privacy. Therefore, the ethics committee decided that informed consent was not required. The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Peking Union Medical College Hospital (ethical approval no.: KS 2019560).

Author Contributions

All authors made substantial contributions to the conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work.

Funding

This research was funded by the Beijing Key Clinical Specialty for Laboratory Medicine-Excellent Project (Grant No. ZK201000).

Disclosure

The authors report no conflicts of interest in relation to this study.

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