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Analytical and clinical validation of multiplex droplet digital PCR assay for detecting pathogenic fungal infection in lungs

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ABSTRACT

Pulmonary invasive fungal infection in immunocompromised hosts is difficult to diagnose, and current tools for diagnosis or monitoring of response to antifungal treatments have inherent limitations. Droplet digital PCR (ddPCR) has emerged as a promising tool for pulmonary pathogen detection with high sensitivity. This study presents a novel ddPCR panel for rapid and sensitive identification of pulmonary fungal pathogens. First, a ddPCR method for detecting three fungal genera, including *Pneumocystis*, *Aspergillus*, and *Cryptococcus*, was established and evaluated. Then, the clinical validation performance of ddPCR was compared with that of qPCR using 170 specimens, and the 6 specimens with inconsistent results were further verified by metagenomics next-generation sequencing, which yielded results consistent with the ddPCR findings. Finally, the area under the ROC curve (AUC) was used to evaluate the efficiency of ddPCR. While the qPCR identified 16 (9.41%) cases of *Aspergillus* and 6 (3.53%) cases of *Pneumocystis*, ddPCR detected 20 (11.76%) *Aspergillus* cases and 8 (4.71%) *Pneumocystis* cases. The AUC for *Aspergillus*, *Cryptococcus*, and *Pneumocystis* was 0.974, 0.998, and 0.975, respectively. These findings demonstrated that the ddPCR assay is a highly sensitive method for identifying pathogens responsible for invasive fungal pulmonary infections, and is a promising tool for early diagnosis.

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Droplet digital PCR (ddPCR); invasive fungal infection; pulmonary; *Aspergillus*; *Pneumocystis*

1. Introduction

Invasive fungal infections affect approximately 6 out of 100,000 people, annually. However, it is estimated that only half of such infections are detected during the patient's lifetime, making invasive fungal infection one of the more commonly overlooked causes of death in intensive-care patients (von Lilienfeld-Toal et al. 2019; Ratemo and Denning 2023). Current microbiological diagnostics frequently fail to identify pathogens (Aguilar-Guisado et al. 2011) due to the inhibitory effect of antimicrobial pretreatment on culture growth and limited preselected targets of multiplex assays.

The opportunistic fungal pathogen *Aspergillus* has attracted increasing attention owing to the detection of a wide array of *Aspergillus* species in aspergillosis patients (Fang and Latgé 2018). The predominant *Aspergillus* species detected in aspergillosis patients (Lockhart et al. 2011; Vivek-Ananth et al. 2018) include *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, and *Aspergillus terreus*, which have been frequently detected in the respiratory tract of cystic fibrosis (CF) patients (de Valk et al. 2009), chronic obstructive pulmonary disease (COPD) patients (Everaerts et al. 2018), and lung transplant recipients (Wu et al. 2023). Moreover, *Aspergillus* spp. can also cause chronic, non-invasive infections classified as chronic pulmonary aspergillosis. *Pneumocystis* is an opportunistic fungal pathogen, presenting clinical importance in non-HIV immunocompromised patients, and *Pneumocystis* pneumonia (PCP) has been a leading cause of opportunistic fungal infection and mortality in HIV-infected persons (Bonnet et al. 2005). Currently, patients without HIV infection account for the majority of cases of PCP in industrialised countries (Mikaelsson et al. 2006), and *Pneumocystis* infections remain a serious public health

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issue. *Pneumocystis jirovecii* is characterised by strong tropism for lungs and airborne host-host transmission (Nevez et al. 2020). *Cryptococcus neoformans* is an encapsulated fungus that widely exists in soil and is a causative agent for primary pulmonary infection (Srikanta et al. 2014). It can cause a severe immune response, especially in immunocompromised hosts, and early and accurate diagnosis of pulmonary cryptococcal infection is critical to prevent *C. neoformans* infections (Park et al. 2009; Kaki 2023).

Currently, conventional examination of pulmonary fungal infection consists of culture, microscopic examination, antigen assay, and histopathology (Fang et al. 2023), with culture being the gold standard method. However, culture examination, such as broncho-alveolar lavage fluid (BALF) culture which is commonly used for detecting fungal pathogens in infection, is not a sensitive and specific diagnostic marker and is often time-consuming and requires a large number of clinical specimens (Saha et al. 2009). Microscopic examination is rapid, but might produce unclear findings or result in misdiagnosis. Antigen assay, a non-culture-based method, is widely used in hospitals and mainly includes the galactomannan test (White et al. 2015; Sehgal et al. 2019) and beta-D-glucan test (Badiee et al. 2012; Urabe et al. 2017); however, this technique can underestimate the positive rate and is poor in independent quality control. Pulmonary histopathology is an invasive method often used when other conventional diagnostic methods fail to produce clear results (Wheat et al. 2002). Recently, PCR, including conventional, nested (Hoenigl et al. 2014), and quantitative PCR (Guegan et al. 2018; Springer et al. 2018; Xu et al. 2020), have been used for the detection of pulmonary fungal infection. This method is usually rapid, sensitive, and requires only a small amount of sample, thus making it a promising alternative to the conventional methods for the early diagnosis of pulmonary fungal infection (Setianingrum et al. 2019).

Droplet digital PCR (ddPCR) is a novel technique based on digital PCR (dPCR) with the droplet generation chip, which does not require a standard curve and produces superior quantitative results, especially with trace and mixed samples (Taylor et al. 2017; Poh et al. 2020). Since the COVID-19 pandemic, ddPCR has become the preferred technique for virus detection (Vasudevan et al. 2021). However, research on the application of ddPCR for the diagnosis of invasive fungal infection is limited. For instance, Zhu et al. (2020) successfully demonstrated the sensitivity of ddPCR in detecting fungal pathogens, but they developed the technique only to detect the teliospore of *Tilletia laevis* in the common bunt of wheat. Similarly, Poh et al. (2020) developed a ddPCR method that could detect only *A. fumigatus* and *A. terreus* in respiratory specimens from normal and diseased people. Therefore, in the present study, we developed a multiplex ddPCR panel designed by myself for the detection of three genera of fungal pathogens, including *Pneumocystis* (namely, *Pneumocystis jirovecii*), *Aspergillus* (namely, *A. fumigatus*, *A. flavus*, *A. niger*, *A. terreus*, and *A. nidulans*), and *Cryptococcus* (namely, *C. neoformans* and *C. gattii*), in immunocompromised patients with suspected invasive fungal pulmonary infection, and comprehensively compared the clinical applications of quantitative real-time PCR (qPCR) and ddPCR methods for rapid and accurate detection of fungal pathogens. The inconsistent results obtained were verified by metagenomics next-generation sequencing (mNGS). The findings of the present study demonstrated that our developed multiplex ddPCR method could be a potential tool for the detection of clinically invasive fungal pulmonary infections.

2. Materials and methods

2.1. Patients and samples

We organised a prospective multicenter cohort study at three medical centres, including East Hospital, Jiangxi Provincial People's Hospital, and Qilu Hospital of Shandong University in China. Our study was approved by the Ethics Committee of East Hospital of Tongji University [(2021)Yanshen(063)]. A total of 170 patients with clinically suspected invasive fungal pulmonary infection were registered in our study from December 2020 to December 2021. The diagnosis of possible, probable, and proven invasive fungal pulmonary infections was based on host, clinical, and mycological criteria according to the definitions provided in EORTC/MSG. Informed consent was directly obtained from the patients or their relatives for sample collection. The following clinical information was recorded for each patient: underlying disease (haematological malignancies, autoimmune disease, pneumonia, malignant tumour, diabetes mellitus, and other diseases), radiological signs, and with or without treatments.

Spontaneously expectorated BALF samples from deep cough were collected and examined whenever cultures were obtained on clinical indications (i.e. at the discretion of the attending physician). The samples were anonymised for both patient characteristics and corresponding culture results by using coded identifiers, and stored at 4 °C for a maximum of 24 h before further processing.

2.2. Nucleic acid extraction

The DNA was extracted using DNA Extraction Kit (iGeneTec, Shanghai, China) following the manufacturer's instructions. A total of 0.2 mL of nucleic acid extraction solution (thoroughly mixed before use) and 1 tube of extracted solids were added to the BALF samples in 1.5 mL centrifuge tube (the solids were removed), and vortexed at high speed in a shaker for 5 min. After instant centrifugation, the samples were heated in a dry bath at 100 °C for 5 min, and then centrifuged at 12,000 r/min for 2 min. The supernatant obtained was transferred to a new 1.5 mL tube and stored at – 20 °C until further use.

2.3. Detection of fungal pathogens by ddPCR

Figure 1 illustrates our developed ddPCR method for the detection of fungal pathogens in BALF samples. First, the samples prepared were tested using a self-designed commercial kit (Invasive Fungi Detection Kit targeting Pulmonary Infection, Pilot Gene Tech., Hangzhou, China). Then, ddPCR analysis was performed using 5-fluorescent-channel ddPCR system (Pilot Gene Tech. Hangzhou, China). The whole process included sample preparation (30–40 min), droplet generation (20 min), PCR amplification (60 min) and reading analysis (30 min). In brief, 5 µL of DNA template were added to 10 µL of the ddPCR premix, which comprised detection primers, probes, and necessary components for PCR amplification. The reaction mixture was gently mixed and added into a ready-to-use disposable plastic chip, and about 20,000 water-in-oil emulsion droplets were generated inside the chip using a droplet generator (AD16, Pilot Gene Tech.). Then, the chip was amplified in a thermal cycler (AD16, Pilot Gene Tech.) under the following cycling parameters: 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. Finally, the chip was

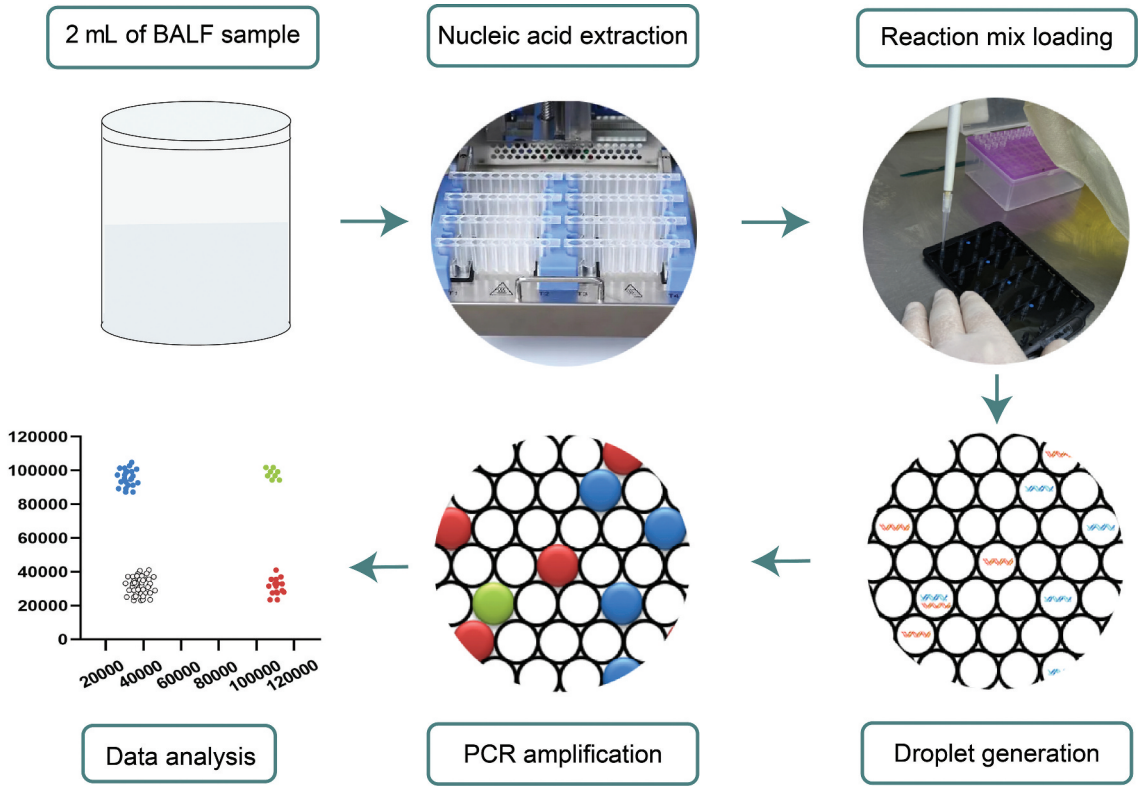

Figure 1. Detection of invasive pulmonary fungal infection by droplet digital PCR (ddPCR).

loaded into a chip scanner (AD16, Pilot Gene Tech.) for fluorescence signal reading and further data analysis.

2.4. Pathogens detection by qPCR

The fungal pathogens were also detected by qPCR assay using the commercial PCR kit. Each reaction mix contained 10 μL of PCR mix, 1 μL of forward and reverse primers, 0.5 μL of probe, and 4 μL of DNA template to obtain a final volume of 20 μL. The reactions were performed with the PCR system under the following conditions: 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 57 °C for 30 s. The result was considered valid only when the cycle threshold (Ct) value of the reference gene was < 40, positive when the Ct values of both the target genes were ≤ 36, and negative when the Ct values of both the target genes were > 36.

2.5. mNGS and data analysis

The BALF specimens were collected from patients according to standard operating procedures. The total DNA was extracted using TIANamp Micro DNA Kit (DP316, Tiangen Biotech, Beijing, China), according to the manufacturer's instructions, and used for DNA library construction through DNA fragmentation (150 bp), end repair, adapter ligation, and unbiased PCR amplification. The Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Lenexa, KS, USA) was utilised for quality control of the DNA libraries (200–300 bp). After the removal of low-complexity reads according to Prinseq (version 0.20.4), the remaining sequences were phylogenetically classified by aligning to PMDB, downloaded from NCBI (ftp.ncbi.nlm.nih.gov/genomes/).

2.6. Limit of blank and limit of detection of ddPCR

A total of 60 negative BALF samples were used as blank control for calculating the limit of blank (LoB), as described in EP17-A2. If the results of the blank samples exhibited normal distribution (*Pneumocystis* and *Cryptococcus*), a parametric approach was used for LoB analysis; otherwise, a nonparametric approach (*Aspergillus*) was employed. The target pathogens were quantified and added to the negative BALF samples for calculating the limit of detection (LoD) of ddPCR, as described in EP17-A2. If LoB = 0, probit regression was used to analyse LoD, and if LoB ≠ 0, a classical approach was utilised for LoD analysis (EP17-A).

2.7. Statistical analysis

The ddPCR data were analysed using Gene DPT software (Pilot Gene Tech.). The threshold for target detection was 3 copies/μL, according to the manufacturers' instructions of the assay panels. The ddPCR was considered as positive if the concentration was higher than the threshold. Comparative analysis was conducted using Pearson χ^2 test, McNemar test, or Fisher exact test (all the tests were 2-tailed), with $P < 0.05$ indicating significant values. The correlation between the Ct values of qPCR and ddPCR was analysed using Spearman correlation test, and $P < 0.05$ (two-sided) was considered to denote statistical significance. All the statistical analyses were performed using GraphPad.

3. Results

3.1. Target pathogens of ddPCR

The target pathogens of ddPCR comprised three fungal genera, which were detected by different fluorescence channels (Table 1). *Pneumocystis* (namely, *P. jirovecii*) was detected by the FAM fluorescence channel; *Aspergillus* (including *A. fumigatus*, *A. flavus*, *A. niger*, *A. terreus*, and *A. nidulans*) was detected by the ROX fluorescence channel; and *Cryptococcus* (namely, *C. neoformans* and *C. gattii*) was detected by the CY5 fluorescence channel.

Table 1. Target pathogens detection for droplet digital PCR (ddPCR).

Fluorescence channel	Genus	Target pathogens
FAM	*Pneumocystis*	*Pneumocystis jiroveci*
ROX	*Aspergillus*	*Aspergillus fumigatus* / *Aspergillus flavus* / *Aspergillus niger* / *Aspergillus terreus* / *Aspergillus nidulans*
CY5	*Cryptococcus*	*Cryptococcus neoformans* / *Cryptococcus gattii*

3.2. Assessment of the specificity of ddPCR

A silicon test using a basic local alignment search tool revealed that all the primers used were highly specific to different strains of *Pneumocystis*, *Aspergillus*, and *Cryptococcus*. In addition, ddPCR examination of 16 kinds of bacteria: *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Enterococcus faecium*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Staphylococcus capitis*, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus hominis*, *Enterobacter cloacae*, *Proteus mirabilis*, *Serratia marcescens*, and *Stenotrophomonas maltophilia*; 28 kinds of fungi: *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, *Candida Krusei*, *Candida guilliermondii*, *Saccharomyces cerevisiae*, *Mucor racemosus*, *Mucor circinelloides*, *Rhizopus arrhizus*, *Trichosporon asahi*, *Pneumocystis jirovecii*, *Nocardia farcinica*, *Nocardia cyriacigeorgica*, *Nocardia abscessus*, *Nocardia brasiliensis*, *Nocardia otitidiscaviarum*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus terreus*, *Aspergillus nidulans*, *Cryptococcus neoformans*, *Cryptococcus gattii*, *Penicillium marneffei*, *Penicillium rubens*, *Penicillium goetzii*, and *Penicillium chrysogenum* by ddPCR, only target pathogens was detected by corresponding fluorescence channel and no cross reaction was found using the primers and probes of this panel. These results demonstrated that the primers and probes of our developed ddPCR panel were highly specific to our target pathogens.

3.3. Evaluation of LoB, LoD, precision, and linearity range of ddPCR

The LoB values for *Pneumocystis*, *Aspergillus*, and *Cryptococcus* were 0, 15, and 0 copies/test, respectively, while the LoD values for the corresponding genera were 3, 35, and 3 copies/test, respectively (Table 2). Precision experiments performed with BALF samples spiked with quantified target pathogens revealed 20 strongly positive and 20 weakly positive samples for each target pathogen. As shown in Figure 2, the variation coefficients of the three fluorescence channels with strong positive samples were ROX = 3.42, FAM = 4.28, and CY5 = 3.12, respectively, while those of the three fluorescence channels with weak positive samples were ROX = 4.95, FAM = 6.16, and CY5 = 3.51, respectively.

Table 2. The result of limit of blank (LoB) and limit of detection (LoD).

Genus	LoB (copies/test)	LoD (copies/test)
Pneumocystis	0	3
Aspergillus	10	35
Cryptococcus	0	3

Ubsequently, the target pathogen suspensions were used as templates to determine the linear range by measuring the optical density (*OD*) of the microbial cells at 600 nm ($0.1 < OD < 1.0$), enumerating the number of microbial cells, calculating the correlation coefficient between the *OD* value for each genus and number of microbial cells, and quantifying the microbial cells of each genus using the *OD* value and correlation coefficient. The ddPCR was performed using two-fold serially diluted microbial suspension for each genus from 20,000 to 600 CFU/mL, and each concentration was tested in triplicate. The results showed that the slope for *Aspergillus*, *P. jirovecii*, and *Cryptococcus* was 1.0145, 0.999, and 0.9999, respectively (Figure 3).

3.4. Comparison of ddPCR and qPCR performance

A total of 170 BALF specimens from 170 patients with suspected invasive pulmonary fungal infection were analysed by ddPCR and qPCR. The ddPCR results showed that 11.76% (20/170) specimens were *Aspergillus*-positive and 88.24% (150/170) specimens were *Aspergillus*-negative, whereas qPCR findings revealed 9.41% (16/170) specimens were *Aspergillus*-positive and 90.59% (154/170) specimens were *Aspergillus*-negative. Furthermore, 4.71% (8/170) and 95.29% (162/170) specimens were found to be *Pneumocystis*-positive and *Pneumocystis*-negative using ddPCR, respectively, while 3.53% (6/170) and 94.67% (164/170) specimens were noted to be *Pneumocystis*-positive and *Pneumocystis*-negative using qPCR, respectively.

The positive coincidence rate between ddPCR and qPCR was 80% for *Aspergillus* detection and 66.67% for *Pneumocystis* detection, whereas the negative coincidence rate between the two methods was 100% for both *Aspergillus* and *Pneumocystis* detection. The κ value between ddPCR and qPCR for *Aspergillus* and *Pneumocystis* detection was 87.59% and 85.11%, respectively (Table 3). The six specimens with inconsistent results between ddPCR and

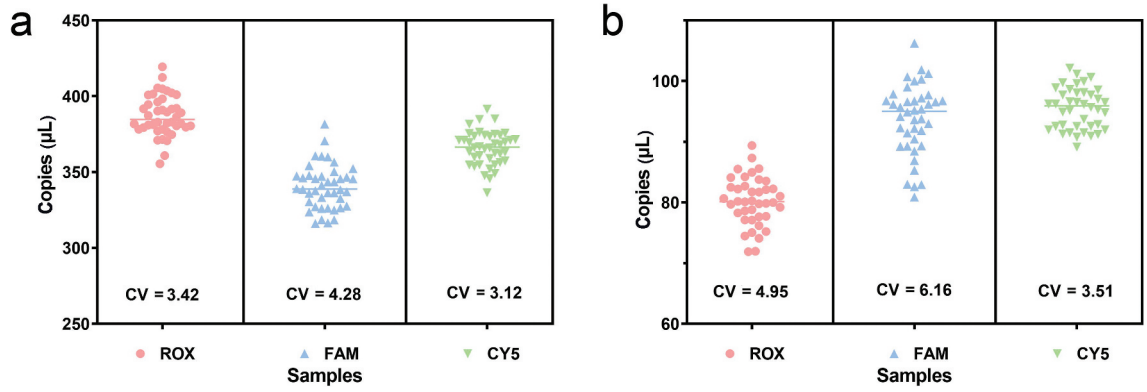

Figure 2. Specificity of droplet digital PCR (ddPCR) in detecting the target pathogen DNA. (a) Strong positive. (b) Weak positive.

Table 3. The coincidence rate of detection results detected by Droplet digital PCR (ddPCR) and quantitative real-time PCR (qPCR).

	Positive coincidence (%)	Negative coincidence (%)	K (%)
Aspergillus	80.00	100.00	87.59
Pneumocystis	66.67	100.00	85.11

Table 4. The inconsistent results between droplet digital PCR (ddPCR) and quantitative real-time PCR (qPCR) were verified by metagenomics next generation sequencing (mNGS).

Specimen No.	ASP DNA for ddPCR (copies/test)	PJ DNA for ddPCR (copies/test)	ASP DNA for qPCR (Ct value)	PJ DNA for qPCR (Ct value)	mNGS
Patient 1	51	0	Undetermined	Undetermined	Aspergillus-positive
Patient 2	42	0	Undetermined	Undetermined	Aspergillus-positive
Patient 3	46.5	0	Undetermined	Undetermined	Aspergillus-positive
Patient 4	43.5	0	Undetermined	Undetermined	Aspergillus-positive
Patient 5	3.5	31.5	Undetermined	Undetermined	Pneumocystis-positive
Patient 6	2.75	27	Undetermined	Undetermined	Pneumocystis-positive

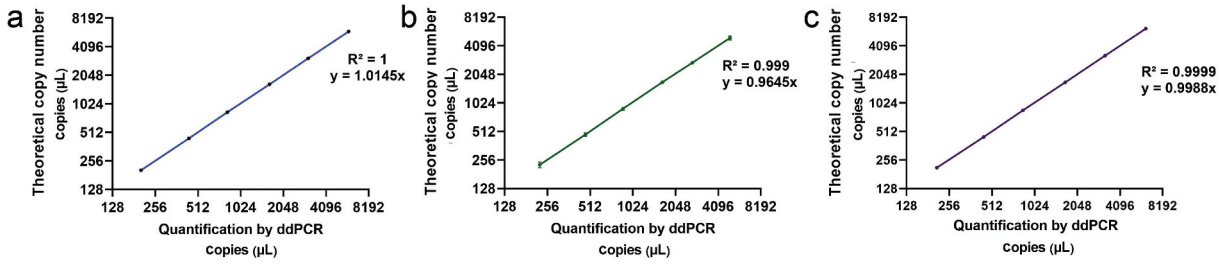

Figure 3. Dynamic target pathogen DNA detection range of droplet digital PCR (ddPCR). (a) *Aspergillus*. (b) *Pneumocystis jirovecii*. (c) *Cryptococcus*.

qPCR were further verified by mNGS, and the results revealed that patients 1–4 were *Aspergillus*-positive, whereas patients 5 and 6 were *Pneumocystis*-positive (Table 4), which were consistent with the ddPCR results.

3.5. Evaluation of the fungal pathogen detection efficiency of ddPCR

A total of 154 specimens from 126 patients with clinical diagnosis data were collected for ddPCR detection, and the receiver operating characteristic (ROC) methodology was used for the analysis of the results. The area under the ROC curve (AUC) for *Aspergillus* genus was 0.974 [95% confidence interval (CI) = 0.995–1; $P < 0.0001$] and the optimal cut-off was 36.8 copies/test, with 84.6% sensitivity and 98.3% specificity; the AUC for *Cryptococcus* genus was 0.998 (95% CI = 0.955–0.993; $P < 0.0001$) and the optimal cut-off was 2.105 copies/test, with 100% sensitivity and 97.9% specificity; and the AUC for *Pneumocystis* genus was 0.975 (95% CI = 0.921–1; $P < 0.0001$) and the optimal cut-off was 5.22 copies/test, with 95.2% sensitivity and 100% specificity (Figure 4).

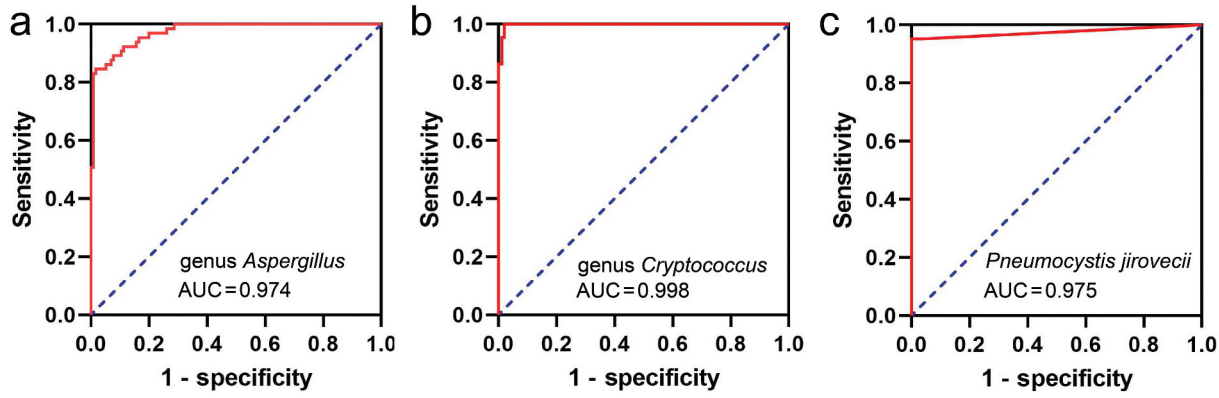

Figure 4. The ROC curve for target pathogen DNA detection by droplet digital PCR (ddPCR). (a) *Aspergillus*. (b) *Cryptococcus*. (c) *Pneumocystis jirovecii*.

4. Discussions

With an increasing number of patients at risk of invasive fungal infections and associated high mortality, there is an urgent need for the development of rapid and accurate diagnostic tools. Molecular techniques, including DNA probes (Laurent et al. 1996), PCR-RFLP molecular analysis (Brown et al. 2004), and DNA sequencing (Roth et al. 2003), have achieved rapid and precise identification of pathogens. Although conventional PCR presents higher sensitivity than microscopic observation, it possesses low specificity and low positive predictive value, which limits its application in clinical practice (Fujisawa et al. 2009). ddPCR allows precise quantification of nucleic acids, facilitating measurement of small percentage differences and quantification of rare variants. Moreover, ddPCR produces more reproducible results and is less susceptible to inhibition than qPCR. In the present study, we established a ddPCR method for the detection of three fungal genera in respiratory specimens and evaluated its analytical performance in identifying the DNA of the target pathogen. The TaqMan primer and probe sets were designed and their specificities were verified, and the primer probe of a single genus was detected by multiple templates. After amplification, only the channel with the primer probe presented positive droplets, and the contrast between the positive droplet and negative droplet was not significantly different from that of the system with the corresponding single template. Furthermore, with respect to multiple primer probes with a single template, only the channel where the template was added exhibited positive droplets after amplification, and the contrast between the positive droplet and negative droplet showed no difference from that of the single primer probe with a single template, indicating that the amplification between different channels did not affect each other and there was no cross-hybridisation. Moreover, the application of our developed ddPCR system to detect the genomic DNAs of some common pulmonary invasive bacterial and fungal pathogens produced negative test results, demonstrating that the probe did not show cross-hybridisation (data not shown). These findings revealed that our developed ddPCR method has high specificity.

Aspergillus has emerged as one of the most common causes of infectious death in severely immunocompromised patients (Latgé and Chamilos 2019). Among chronic lung diseases, COPD is associated with the highest *Pneumocystis* colonisation, reaching 37%–55% (Calderón et al. 2007). The diagnosis of *Pneumocystis* pneumonia mostly depends on the microscopic evidence of the characteristic organism in the respiratory specimens such as BALF or induced BALF (Catherinot et al. 2010).

In the present study, comparison of the clinical performance of our developed ddPCR method with that of qPCR in detecting three pathogenic fungal genera in BALF specimens showed that ddPCR and qPCR consistently detected 97.65% (166/170) *Aspergillus*-positive BALF specimens and 98.82% (168/170) *Pneumocystis*-positive BALF specimens, implying that both these methods exhibit highly coherent results; however,

ddPCR exhibited higher detection rate than qPCR (Table 3). The six BALF specimens that presented inconsistent results were further examined by mNGS, and the findings were consistent with the ddPCR results (Table 4). As the qPCR test kit did not cover *Cryptococcus* and *Nocardia*, the positive and negative coincidence rates were not included in this study (Zheng et al. 2021). Furthermore, ddPCR and qPCR were coherent in detecting 78.37% (29/37) *P. jirovecii*-positive BALF samples, with ddPCR capable of detecting very low levels of *P. jirovecii* which were undetectable by qPCR.

In addition, we also investigated the clinical performance of ddPCR method through ROC curve (Figure 3), and the cut-off value for ddPCR was confirmed using ROC curve analysis. The AUC and cut-off value indicated that the accuracy of ddPCR is very high. A previous research on in-time detection of pathogens in the early stage of sepsis in two cases using ddPCR showed that ddPCR is clinically valuable owing to its high sensitivity and short turnaround time, facilitating ultra-early diagnosis and guidance of medication (Lin et al. 2022). Furthermore, evaluation of the development and clinical validation of a ddPCR method for the detection of *Acinetobacter baumannii* and *Klebsiella pneumonia* suggested that ddPCR was sensitive and rapid in identifying these pathogens with a specificity of 100% for each strain (Zheng et al. 2021). Similarly, Zhou et al. (2021) also demonstrated that ddPCR assay is a rapid and sensitive method for detecting infections with short turnaround time and high sensitivity, which could be very useful for early diagnosis of infections and treatment optimisation.

5. Conclusions

In conclusion, we developed a novel ddPCR method to detect four major fungal pathogens in patients with suspected invasive pulmonary fungal infection. Clinical validation revealed that our developed method outperformed the qPCR method in specificity, sensitivity, and turnaround time, serving as a promising technique for early and accurate diagnosis of invasive pulmonary disease. However, this pilot study only included four pathogens responsible for invasive pneumonia; therefore, more clinically important pathogens must be investigated in future studies.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Ethical approval

The protocol was approved by the Ethics Committee of the National Institute for Communicable Disease Control and Prevention of the Chinese Center for Disease Control and Prevention.

Author contributions

Jian Guo, conception and design. Wenjie Tian and Huiping Lin, data analysis and manuscript writing. Hui Chen and Liang Hu, revising the work. Xuejuan Gao, Jiang Xia, Hao Yu, collection and assembly of data. Wenjuan Wu and Wei Li, final approval of the manuscript and agreement to be accountable for all aspects of the work.

References

Aguilar-Guisado M, Jiménez-Jambrina M, Espigado I, Rovira M, Martino R, Oriol A, Borrell N, Ruiz I, Martín-Dávila P, de la Cámara R, et al. 2011. Pneumonia in allogeneic stem cell transplantation recipients: A multicenter prospective study. Clin Transplant. 25(6):E629–638. doi: 10.1111/j.1399-0012.2011.01495.x.

Badiee P, Alborzi A, Karimi M, Pourabbas B, Haddadi P, Mardaneh J, Moieni M. 2012. Diagnostic potential of nested PCR, galactomannan EIA, and beta-D-glucan for invasive aspergillosis in pediatric patients. J Infect Dev Ctries. 6(4):352–357. doi: 10.3855/jidc.2110.

Bonnet F, Lewden C, May T, Heripret L, Jougla E, Bevilacqua S, Costagliola D, Salmon D, Chêne G, Morlat P. 2005. Opportunistic infections as causes of death in HIV-infected patients in the HAART era in France. Scand J Infect Dis. 37(6–7):482–487. doi: 10.1080/00365540510035328.

Brown JM, Pham KN, McNeil MM, Lasker BA. 2004. Rapid identification of *Nocardia farcinica* clinical isolates by a PCR assay targeting a 314-base-pair species-specific DNA fragment. J Clin Microbiol. 42(8):3655–3660. doi: 10.1128/jcm.42.8.3655-3660.2004.

Calderón EJ, Rivero L, Respaldiza N, Morilla R, Montes-Cano MA, Friaza V, Muñoz-Lobato F, Varela JM, Medrano FJ, Horra

Cde L. 2007. Systemic inflammation in patients with chronic obstructive pulmonary disease who are colonized with *Pneumocystis jiroveci*. Clin Infect Dis. 45(2):e17–19. doi: 10.1086/518989.

Catherinot E, Lanternier F, Bougnoux ME, Lecuit M, Couderc LJ, Lortholary O. 2010. *Pneumocystis jirovecii* pneumonia. Infect Dis Clin North Am. 24(1):107–138. doi: 10.1016/j.idc.2009.10.010.

De Valk HA, Klaassen CH, Yntema JB, Hebestreit A, Seidler M, Haase G, Müller FM, Meis JF, de Valk HA. 2009. Molecular typing and colonization patterns of *Aspergillus fumigatus* in patients with cystic fibrosis. J Cyst Fibros. 8(2):110–114. doi: 10.1016/j.jcf.2008.10.003.

Everaerts S, Lagrou K, Vermeersch K, Dupont LJ, Vanaudenaerde BM, Janssens W. 2018. *Aspergillus fumigatus* detection and risk factors in patients with COPD–bronchiectasis overlap. Int J Mol Sci. 19(2):523. doi: 10.3390/ijms19020523.

Fang W, Latgé JP. 2018. Microbe profile: *Aspergillus fumigatus*: A saprotrophic and opportunistic fungal pathogen. Microbiol. 164(8):1009–1011. doi: 10.1099/mic.0.000651.

Fang W, Wu J, Cheng M, Zhu X, Du M, Chen C, Liao W, Zhi K, Pan W. 2023. Diagnosis of invasive fungal infections: Challenges and recent developments. J Biomed Sci. 30 (1):42. doi: 10.1186/s12929-023-00926-2.

Fujisawa T, Suda T, Matsuda H, Inui N, Nakamura Y, Sato J, Toyoshima M, Nakano Y, Yasuda K, Gemma H, et al. 2009. Real-time PCR is more specific than conventional PCR for induced sputum diagnosis of *Pneumocystis pneumonia* in immunocompromised patients without HIV infection. Respirology. 14(2):203–209. doi: 10.1111/j.1440-1843.2008.01457.x.

Guegan H, Robert-Gangneux F, Camus C, Belaz S, Marchand T, Baldeyrou M, Gangneux JP. 2018. Improving the diagnosis of invasive aspergillosis by the detection of *Aspergillus* in broncho-alveolar lavage fluid: Comparison of non-culture-based assays. J Infect. 76(2):196–205. doi: 10.1016/j.jinf.2017.11.011.

Hoenigl M, Prattes J, Spiess B, Wagner J, Prueller F, Raggam RB, Posch V, Duettmann W, Hoenigl K, Wölfler A, et al. 2014. Performance of galactomannan, beta-d-glucan, *Aspergillus* lateral-flow device, conventional culture, and PCR tests with bronchoalveolar lavage fluid for diagnosis of invasive pulmonary aspergillosis. J Clin Microbiol. 52(6):2039–2045. doi: 10.1128/JCM.00467-14.

Kaki R. 2023. Risk factors and mortality of the newly emerging *Candida auris* in a university hospital in Saudi Arabia. Mycology. 14(3):256–263. doi: 10.1080/21501203.2023.2227218.

Latgé JP, Chamilos G. 2019. *Aspergillus fumigatus* and aspergillosis in 2019. Clin Microbiol Rev. 33(1):e00140–18. doi: 10.1128/CMR.00140-18.

Laurent F, Carlotti A, Boiron P, Villard J, Freney J. 1996. Ribotyping: A tool for taxonomy and identification of the *Nocardia asteroides* complex species. J Clin Microbiol. 34 (5):1079–1082. doi: 10.1128/jcm.34.5.1079-1082.1996.

Lin K, Zhang HC, Zhao YH, Xia J, Ai JW, Zhang WH. 2022. The direct application of plasma droplet digital PCR in the ultra-early pathogen detection and warning during sepsis: Case reports. J Infect Public Health. 15(4):450–454. doi: 10.1016/j.jiph.2022.03.008.

Lockhart SR, Zimbeck AJ, Baddley JW, Marr KA, Andes DR, Walsh TJ, Kauffman CA, Kontoyiannis DP, Ito JI, Pappas PG, et al. 2011. *In vitro* echinocandin susceptibility of *Aspergillus* isolates from patients enrolled in the transplant-associated infection surveillance network. Antimicrob Agents Chemother. 55(8):3944–3946. doi: 10.1128/aac.00428-11.

Mikaelsson L, Jacobsson G, Andersson R. 2006. Pneumocystis pneumonia–A retrospective study 1991-2001 in Gothenburg, Sweden. J Infect. 53(4):260–265. doi: 10.1016/j.jinf.2005.06.014.

Nevez G, Hauser PM, Le Gal S. 2020. *Pneumocystis jirovecii*. Trends Microbiol. 28(12):1034–1035. doi: 10.1016/j.tim.2020.03.006.

Park BJ, Wannemuehler KA, Marston BJ, Govender N, Pappas PG, Chiller TM. 2009. Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. Aids. 23(4):525–530. doi: 10.1097/QAD.0b013e328322ffac.

Poh TY, Tiew PY, Lim AYH, Thng KX, Binte Mohamed Ali NA, Narayana JK, Mac Aogáin M, Tien Z, Chew WM, Wai Chan AK, et al. 2020. Increased chitotriosidase is associated with *Aspergillus* and frequent exacerbations in South-East Asian patients with bronchiectasis. Chest. 158(2):512–522. doi: 10.1016/j.chest.2020.02.048.

Ratemo SN, Denning DW. 2023. Burden of fungal infections in Kenya. Mycology. 14(2):142–154. doi: 10.1080/21501203.2023.2204112.

Roth A, Andrees S, Kroppenstedt RM, Harmsen D, Mauch H. 2003. Phylogeny of the genus *Nocardia* based on reassessed 16S rRNA gene sequences reveals underspeciation and division of strains classified as *Nocardia asteroides* into three established species and two unnamed taxons. J Clin Microbiol. 41(2):851–856. doi: 10.1128/jcm.41.2.851-856.2003.

Saha DC, Xess I, Biswas A, Bhowmik DM, Padma MV. 2009. Detection of *Cryptococcus* by conventional, serological and molecular methods. J Med Microbiol. 58(Pt 8):1098–1105. doi: 10.1099/jmm.0.007328-0.

Sehgal IS, Dhooria S, Choudhary H, Aggarwal AN, Garg M, Chakrabarti A, Agarwal R. 2019. Utility of serum and bronchoalveolar lavage fluid galactomannan in diagnosis of chronic pulmonary aspergillosis. J Clin Microbiol. 57(3): e01821–18. doi: 10.1128/jcm.01821-18.

Setianingrum F, Rautemaa-Richardson R, Denning DW. 2019. Pulmonary cryptococcosis: A review of pathobiology and clinical aspects. Med Mycol. 57(2):133–150. doi: 10.1093/mmy/myy086.

Springer J, White PL, Kessel J, Wieters I, Teschner D, Korczynski D, Liebregts T, Cornely OA, Schwartz S, Elgeti T, et al. 2018. A comparison of *Aspergillus* and mucorales PCR testing of different bronchoalveolar lavage fluid fractions from patients with suspected invasive pulmonary fungal

disease. J Clin Microbiol. 56(2):e01655–17. doi:10.1128/JCM.01655-17.

Srikanta D, Santiago-Tirado FH, Doering TL. 2014. *Cryptococcus neoformans*: Historical curiosity to modern pathogen. Yeast. 31(2):47–60. doi: 10.1002/yea.2997.

Taylor SC, Laperriere G, Germain H. 2017. Droplet digital PCR versus qPCR for gene expression analysis with low abundant targets: From variable nonsense to publication quality data. Sci Rep. 7(1):2409. doi: 10.1038/s41598-017-02217-x.

Urabe N, Sakamoto S, Sano G, Suzuki J, Hebisawa A, Nakamura Y, Koyama K, Ishii Y, Tateda K, Homma S, et al. 2017. Usefulness of two *aspergillus* PCR assays and *Aspergillus* galactomannan and β-d-glucan testing of bronchoalveolar lavage fluid for diagnosis of chronic pulmonary aspergillosis. J Clin Microbiol. 55(6):1738–1746. doi: 10.1128/jcm.02497-16.

Vasudevan HN, Xu P, Servellita V, Miller S, Liu L, Gopez A, Chiu CY, Abate AR. 2021. Digital droplet PCR accurately quantifies SARS-CoV-2 viral load from crude lysate without nucleic acid purification. Sci Rep. 11(1):780. doi: 10.1038/s41598-020-80715-1.

Vivek-Ananth RP, Mohanraj K, Vandanashree M, Jhingran A, Craig JP, Samal A. 2018. Comparative systems analysis of the secretome of the opportunistic pathogen *Aspergillus fumigatus* and other *Aspergillus* species. Sci Rep. 8(1):6617. doi: 10.1038/s41598-018-25016-4.

von Lilienfeld-Toal M, Wagener J, Einsele H, Cornely OA, Kurzai O. 2019. Invasive fungal infection. Dtsch Arztebl Int. 116(16):271–278. doi: 10.3238/arztebl.2019.0271.

Wheat LJ, Goldman M, Sarosi G. 2002. State-of-the-art review of pulmonary fungal infections. Semin Respir Infect. 17 (2):158–181. doi: 10.1053/srin.2002.33685.

White PL, Wingard JR, Bretagne S, Löffler J, Patterson TF, Slavin MA, Barnes RA, Pappas PG, Donnelly JP. 2015. *Aspergillus* polymerase chain reaction: Systematic review of evidence for clinical use in comparison with antigen testing. Clin Infect Dis. 61(8):1293–1303. doi: 10.1093/cid/civ507.

Wu J, Li C, Gao P, Zhang C, Zhang P, Zhang L, Dai C, Zhang K, Shi B, Liu M, et al. 2023. Intestinal microbiota links to allograft stability after lung transplantation: A prospective cohort study. Sig Transduct Target Ther. 8(1):326. doi: 10.1038/s41392-023-01515-3.

Xu T, Yao Z, Liu J, Zhang H, Din GMU, Zhao S, Chen W, Liu T, Gao L. 2020. Development of droplet digital PCR for the detection of *Tilletia laevis*, which causes common bunt of wheat, based on the SCAR marker derived from ISSR and real-time PCR. Sci Rep. 10(1):16106. doi: 10.1038/s41598-020-72976-7.

Zheng Y, Jin J, Shao Z, Liu J, Zhang R, Sun R, Hu B. 2021. Development and clinical validation of a droplet digital PCR assay for detecting *Acinetobacter baumannii* and *Klebsiella pneumoniae* in patients with suspected bloodstream infections. Microbiologyopen. 10(6):e1247. doi: 10.1002/mbo3.1247.

Zhou F, Sun S, Sun X, Chen Y, Yang X. 2021. Rapid and sensitive identification of pleural and peritoneal infections by droplet digital PCR. Folia Microbiol (Praha). 66(2):213–219. doi: 10.1007/s12223-020-00834-0.

Zhu G, Yang Y, Wang H, Xie J, Hu J, Guo W, Zhang L, Liu Z, Chen X, Chang J, et al. 2020. Monitoring treatment-free remission by droplet digital PCR in CML patients with deep molecular response to tyrosine kinase inhibitor: An analysis based on real-world data. Ann Clin Lab Sci. 50(5):591–599. PMID: 33067205.