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Research article

Evaluation of a PCR-based lateral flow device for detecting *Aspergillus* and *Candida* species from clinical specimens

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ABSTRACT

Objectives: The diagnosis of invasive fungal diseases (IFDs) is time consuming and lacks sensitivity. In this research a rapid and easy to use immunochromatography-based DNA biosensor system was developed to detect *Candida* and *Aspergillus* pathogens at genus level, while specifically detecting *Candida glabrata*, *Candida krusei* and *Aspergillus terreus*. This system combines multiplex PCR with a lateral flow assay (LFA) dipstick.

Methods: Three separate multiplexed PCR reactions were designed together with a testing algorithm, using biotin, digoxigenin and Tamra fluorophore-labelled fungal internal transcribed spacer universal fungal primers, fungal genera-specific primers, and species-specific primers to produce labelled PCR products that were detected on the LFA dipstick. The LFA dipstick, in a modified sandwich format, utilises immobilised antibodies complementary to the fluorophore labels on the PCR products, and gold nanoparticles to form a visible red line that indicates the presence of the targeted fungus. To validate the developed system, 203 clinical samples suspected of fungal infection were collected from two hospitals in Kuala Lumpur and tested.

Results: The limits of detection of the multiplexed PCR were in the range of 5–100 CFU/mL for fungal spiked human blood samples. Against the clinical diagnosis of proven or probable IFDs, the findings show that the LFA system produced a high specificity of 99.4 % while the sensitivity was only moderate at 47.8 % due to the difficulty of extracting fungal DNA from blood samples. The positive and negative predictive values however were promising at 91.7 % and 93.7 %, respectively.

Conclusion: The developed LFA system has great potential for further refinement to be used as a new tool in the detection of IFDs.

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1. Introduction

The global incidence of invasive fungal diseases (IFDs) is estimated to affect nearly 1.9 million patients annually, with a considerable number of these cases being life-threatening and resulting in over 1.6 million deaths each year [1]. These infections which lead to substantial morbidity and mortality and significant economic burden are mainly caused by *Candida* and *Aspergillus* genera [2]. Traditionally, IFDs are diagnosed using conventional cultures, serology, histopathology, and radiology. However, many advances have been made in diagnostic mycology including serology-based diagnostics, molecular based diagnostics, and biosensor-based assays [3].

The lateral flow assay (LFA) is a rapid test technique that is based on the principles of paper or membrane chromatography and can be read with the naked eye by observing the color change of the tracer chemical. While this detection strategy is simple and fast, the results are qualitative, lacking sensitivity, and may only work for certain applications [4]. On the other hand, testing using polymerase chain reaction (PCR) can be highly sensitive and specific but is relatively slower than LFA [3]. Combining these two methods could result in a test that is highly sensitive, specific, and rapid. Therefore, we developed and evaluated an LFA-based biosensor to detect the presence of *Candida* or *Aspergillus* DNA from PCR of clinical specimens.

Detection of pathogens in clinical specimens by PCR and sequencing is most desirable for confirmation of identification. However, the tests can be laborious, time consuming and costly. Therefore, we attempted to bypass electrophoresis and sequencing by using the lateral flow assay to identify the PCR product.

2. Methods

2.1. Study design and site

This was a cross-sectional study involving clinical specimens of patients who were admitted at two hospitals in Kuala Lumpur, Malaysia: Hospital Kuala Lumpur (HKL) and Hospital Canselor Tuanku Muhriz (HCTM).

2.2. Sample size calculation

The sample size was calculated according to the formula by Cochran (1963) as follows:

$$\mathrm{n} = \frac{z^2\, X\, P(1-P)}{\Delta^2}$$

Where Z-score = 1.96 (for 95 % confidence interval); $\Delta = 0.1$ (10 % precision); and P = 0.5 (we took the prevalence of 50 % to give us the largest sample size because the prevalence of IFD in Malaysia was unknown at the time this study was designed).

$$\text{Therefore, } \mathrm{n} = \frac{1.96^2\, X\, 0.5(1-0.5)}{0.1^2} = 182.5$$

Considering a 10 % drop-out rate (182.5/0.9), a sample size of 203 was finally determined.

2.3. Inclusion and exclusion criteria

The specimens included in this study were obtained from adult patients (18 years and above) with clinical suspicion of IFDs. Pregnant women and children (less than 18 years) were excluded. The patients were categorized as follows: 1) Proven and probable IFDs according to EORTC criteria. These patients' samples may be considered as true positives for IFDs; 2) Negative for IFDs according to EORTC criteria. These patients' samples may be considered as true negatives for IFDs; and 3) Possible IFDs according to EORTC criteria. These patients IFD status may be further elucidated with the DNA detection assay used.

2.4. Sample collection

Following ethics approval and written informed consent from participating patients, clinical specimens were collected from the patients in the wards of HKL and HCTM according to standard clinical practice. The various types of specimens, included blood, pleural fluid, bronchial washings, peritoneal fluid, bronchoalveolar lavage, cerebrospinal fluid, serum, tissue, and abdominal drain fluid, were sent to the laboratory for further investigations of infective fungal diseases (polymerase chain reaction and lateral flow assay).

2.5. Extraction of DNA from clinical specimens

DNA extraction from 3 to 5 mL whole blood and spiked blood samples was performed using a combination of two kits, Promega Wizard Genomic DNA Extraction Kit (Promega, Wisconsin) and Qiagen Biostic DNA Extraction Kit (Qiagen, Hilden), while DNA from other specimens were extracted using Qiagen Biostic DNA extraction kit with bead beating (Qiagen, Hilden), based on manufacturers' recommended procedures. For blood samples, initially the samples were extracted with the Qiagen Biostic DNA Extraction Kit which is intended for the extraction of microbial DNA from blood culture, however this kit does not include steps for the removal of human DNA. As excess amounts of human DNA may interfere with the PCR amplification of fungal targets, the DNA extraction protocol was

modified to remove human DNA using the Promega kit which involved multiple lysis steps using Promega Cell Lysis and Promega Nuclei Lysis solutions to lyse human cells, followed by centrifugation to remove human DNA from the supernatant. The final resulting pellet, which should contain any fungal cells present in the sample, was extracted using bead beating and the Qiagen Biostic DNA Extraction Kit. All extracted DNA from clinical specimens was stored for the multiplex PCR assay amplification.

2.6. Polymerase chain reaction (PCR)

Three sequential multiplex PCR assays were designed for the specific amplification of the universal fungal gene, *Candida* genes, and *Aspergillus* genes. The first multiplex PCR utilized the universal fungal primer ITS1 (biotin labelled) as forward primer to pair with either ITS4 (digoxigenin labelled) or a reverse primer for *Candida* genus (TAMRA labelled). The second multiplex PCR was for detection of two *Candida* species, *C. glabrata* and *C. krusei*. The forward primer was ITS 1 (biotin labelled) to pair with either primer for *C. glabrata* (digoxigenin labelled) or *C. krusei* (TAMRA labelled) for PCR amplification. The third multiplex PCR was for detection of *Aspergillus* genus and *A. terreus* species. The forward primer was ITS 1 (biotin labelled) to pair with either primer for *Aspergillus* genus (digoxigenin labelled) or *A. terreus* (TAMRA labelled). A table of the expected PCR products of each multiplex assay is listed in supplementary tables (A, B, C). PCR products of each multiplex assay were analysed on the LFA strip, as well as on agarose gel electrophoresis.

2.7. Lateral flow assay

A lateral flow assay (LFA) was developed to detect multiplex PCR products of universal fungal DNA, *Aspergillus* and *Candida* genera and species of *A. terreus*, *C. glabrata* and *C. krusei* in a two-step testing procedure. The LFA strips were produced at Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia, Kubang Kerian, Malaysia. The reaction pad was lined with anti-digoxigenin, anti-TAMRA antibodies as the capture lines which capture PCR amplicons labelled with digoxigenin, and TAMRA haptens respectively. Control line was lined with anti-streptavidin. Streptavidin-conjugated 40 nm gold nanoparticles were immobilised on the conjugate pad of the LFA.

A representative diagram of the LFA is shown in Fig. 1. B-Line captures TAMRA labelled PCR products, while A-Line captures digoxigenin-labelled PCR products. Captured PCR products on the LFA produced visible red lines from streptavidin-conjugated nanogold particles that bind to the biotin-labelled PCR products. Excess streptavidin-conjugated nanogold particles shows up on the internal control line of the LFA and then captured in the absorption pad. A volume of 10 μL PCR products of each multiplex assay

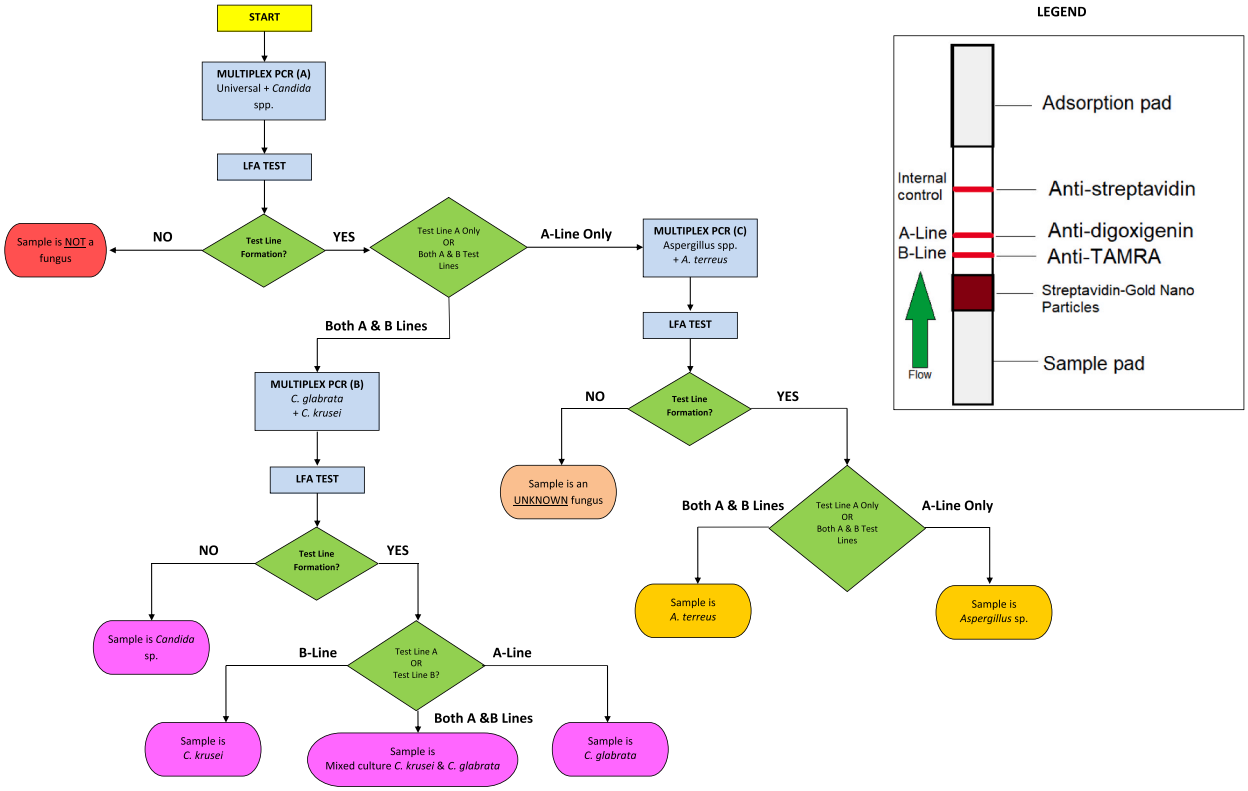

Fig. 1. The algorithm for lateral flow assay (LFA) system to detect invasive fungal infections.

were applied on the LFA strip and lines produced were visually analysed.

The extracted DNA of each specimen was subjected to an algorithm for the multiplex PCR-LFA test for analysis (Fig. 1) which shows the step-wise procedure. Another 10 μL of the same PCR products was analysed via agarose gel electrophoresis. The results of the assay were compared with actual clinical diagnosis of IFDs (proven or probable) according to the European Organisation for Research and Treatment of Cancer/Mycosis Study Group Education and Research Consortium (EORTC/MSGERC) criteria [5] and the laboratory results from the hospitals where clinical samples were obtained.

A Malaysian patent has been applied for the developed multiplex PCR-LFA algorithm and approval is pending.

3. Results

A total of 203 clinical samples were analysed. Initial testing with fungal spiked blood samples revealed a detection limit of 5–100 cells/mL (results for *C. albicans* shown in Fig. 2). The limits of detection (LOD) of the multiplexed PCR were ~5 CFU/mL for both *C. glabrata* and *C. krusei*; ~50 CFU/mL for *C. albicans*; and 100 CFU/mL for *A. terreus*.

Of the 203 clinical samples, 9.9 % (n = 20) specimens were from proven IFD cases and 1.5 % (n = 3) from probable IFD cases (Table 1). Compared to proven and probable IFDs, the PCR based LFA biosensor showed a specificity of 99.4 % and sensitivity of 47.8 %. While the positive predictive and negative predictive values were 91.7 % and 93.7 % respectively. Accuracy was calculated to be 93.6 % (Table 2). None of the whole blood specimens returned positive multiplex PCR results even though seven were positive for hospital fungal culture. However, one bronchoalveolar lavage (BAL) specimen was positive by PCR despite being negative for culture.

DNA from the 12 specimens that returned positive multiplex PCRs underwent simplex PCR using unlabeled universal fungal ITS-1 and ITS-4 primers and the PCR products were sequenced to verify the LFA test results (Table 3). Based on NCBI BLAST similarity analysis, one specimen (abdominal drain) had a 99.88 % match to *C. glabrata*, five (bronchial washing and serum) had >99 % match to *C. albicans*. Meanwhile, one specimen (serum) had a 99.65 % match to *A. terreus* while three (BAL, pleural and peritoneal fluid) had >99 % matches to *A. fumigatus* (including one BAL negative culture sample). The fungal identification corresponded to the hospital laboratory results. However, another specimen (bronchial washing) showed a low match (73 %) to *Aspergillus*, due to it being a mixed DNA sample while hospital culture results yielded *Geotrichum* sp. Interestingly, one specimen (bronchial washing) gave a 100 % match to *Penicillium* sp which showed non-sporulating hyphae in culture. All these sequence matches agreed with the agarose gel visualisations and LFA test results. An example of gel electrophoresis visualization and corresponding LFA result are shown in Figs. 3 and 4.

4. Discussion

Our preliminary study with fungal-spiked blood culture bottles showed a detection limit comparable to a previous study [6]. The detection sensitivity in our study was limited by the DNA extraction procedure used. At the time this research was conducted, there was no commercial kit available for the extraction of microbial DNA from blood samples which included reagents for removal of human

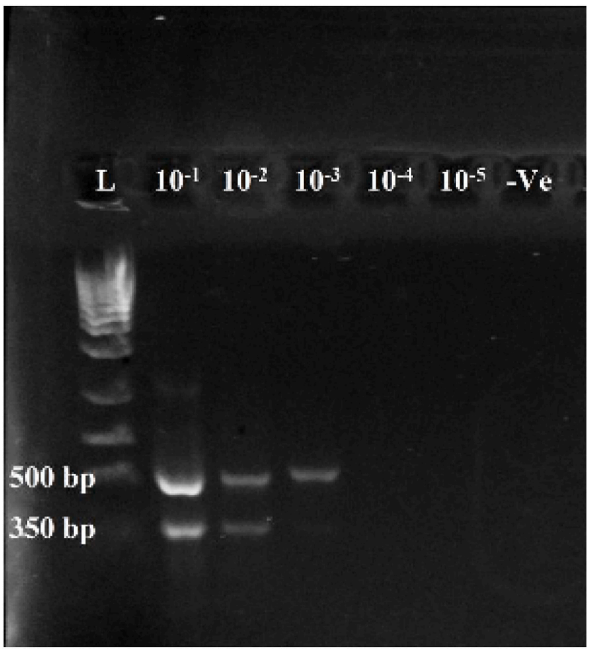

Fig. 2. Agarose gel results of multiplex PCR using DNA extracted from blood samples spiked with serially diluted *C. albicans* cells. The lanes are marked with the respective dilutions. The lane marked –ve is negative control with no DNA. The limit of detection for *C. albicans* is approximately 50 CFU/mL.

Table 1
Specimen types and clinical diagnosis as compared to PCR-LFA results of specimens.

Specimen type	Clinical diagnosis of IFD[a]				PCR- LFA positive (n = 12)
	Proven (%)	Probable (%)	Proven & Probable (%)	Negative (%)	
Blood (n = 44)	7 (15.9)	0 (0)	7 (15.9)	37 (84.1)	0 (0)
Pleural fluid (n = 35)	1 (2.9)	0 (0)	1 (2.9)	34 (97.1)	1 (8.3)
Bronchial washing (n = 29)	8 (27.6)	0 (0)	8 (27.6)	21 (72.4)	5 (41.7)
Peritoneal fluid (n = 25)	2 (8)	0 (0)	2 (8)	23 (92)	2 (16.7)
Bronchoalveolar lavage (BAL) (n = 22)	0 (0)	2 (9.1)	2 (9.1)	20 (90.9)	1 (8.3)
Cerebrospinal fluid (n = 22)	0 (0)	0 (0)	0 (0)	22 (100)	0 (0)
Serum (n = 18)	1 (5.6)	1 (5.6)	2 (11.1)	16 (88.9)	2 (16.7)
Tissue (n = 7)	0 (0)	0 (0)	0 (0)	7 (100)	0 (0)
Abdominal drain (n = 1)	1 (100)	0 (0)	1 (100)	0 (0)	1 (8.3)
All specimens (n = 203)	20 (9.9)	3 (1.5)	23 (11.3)	180 (88.7)	12 (100)
PCR-LFA positive (n = 12)	9 (75)	2 (16.7)	11 (91.7)	1 (8.3)	12 (100)
PCR-LFA negative (n = 191)	11 (5.8)	1 (0.5)	12 (6.3)	179 (93.7)	191 (100)

PCR, polymerase chain reaction; LFA, lateral flow assay.
[a] Clinical diagnosis according to EORTC/MSGERC definition of invasive fungal diseases (IFDs).

Table 2
Performance of PCR-LFA against EORTC diagnosis of IFD.

Statistics[a]	Value	95 % CI
Sensitivity	47.83 %	26.82 %–69.41 %
Specificity	99.44 %	96.94 %–99.99 %
Positive Likelihood Ratio	86.09	11.64 to 636.49
Negative Likelihood Ratio	0.52	0.35 to 0.78
Positive Predictive Value	91.67 %	59.80 %–98.79 %
Negative Predictive Value	93.72 %	90.98 %–95.66 %
Accuracy	93.60 %	89.30 %–96.55 %

[a] calculated using MedCalc software (https://www.medcalc.org/calc/diagnostic_test.php.)

Table 3
Results of DNA sequencing for clinical specimens positive for multiplex PCR and LFA.

Specimen number	Specimen type	NCBI BLAST result of sequenced PCR product	Percentage identity (%)	Identification at hospital laboratory
HUKM-45	Abdominal drain	*Candida glabrata*	99.88	*Candida glabrata*
HUKM-46	Peritoneal fluid	*Candida albicans*	99.44	*Candida albicans*
HUKM-59	Serum	*Aspergillus terreus*	99.65	*Aspergillus* sp.
HUKM-82	Serum	*Candida albicans*	100.00	*Candida albicans*
HKL-1	Bronchial washing	*Candida albicans*	99.60	*Candida albicans*
HKL-8	Bronchial washing	*Aspergillus fumigatus*	73.17	*Geotrichum* sp.
HKL-10	Bronchial washing	*Candida albicans*	99.60	*Candida albicans*
HKL-13	Bronchoalveolar- lavage	*Aspergillus fumigatus*	100.00	NEGATIVE
HKL-16	Bronchial washing	*Candida albicans*	99.80	*Candida albicans*
HKL-52	Bronchial washing	*Penicillium* sp.	100.00	Non-sporulating hyphae
HKL-73	Peritoneal fluid	*Aspergillus fumigatus*	99.65	*Aspergillus fumigatus*
HKL -95	Pleural fluid	*Aspergillus fumigatus*	99.83	*Aspergillus fumigatus*

DNA. When human DNA was present in excess in the sample, non-specific PCR amplification occurred. Hence, we utilized two kits for the purpose of first lysing human cells and removing human DNA from blood samples, followed by extraction of fungal DNA. The protocol was successful when tested with spiked blood samples, however the limit of detection was variable. The extraction of fungal DNA from patients' blood samples was not successful. We postulate that it was due to the low fungal load in blood samples 3[3]. Furthermore, the multiple lysis and centrifugation steps performed to remove blood cells and human DNA may have resulted in unintentional removal of some fungal cells. From our experience, enriched blood samples (following fungal growth in blood culture media) yields far superior results for DNA extraction and PCR detection. The use of a DNA extraction kit specifically for microbial DNA extraction from body fluid and blood, such as the MolYsisTM Complete5 kit (Molzym, Bremen) may increase detection sensitivity.

The developed PCR-LFA algorithm enables detection of all fungal pathogens and genus *Candida* in the first multiplex PCR-LFA assay. As candidiasis is the most commonly occurring IFD, this first test will identify all *Candida* spp. isolates. If the first PCR-LFA test showed positive for both universal fungus and *Candida*, further identification of *Candida* species is possible in a second multiplex PCR-LFA test. On the other hand, if the first PCR-LFA test was positive only for fungus and negative for *Candida*, a second PCR-LFA to detect *Aspergillus* genus and *A. terreus* can be performed. This algorithm lends itself to selective testing according to the initial result

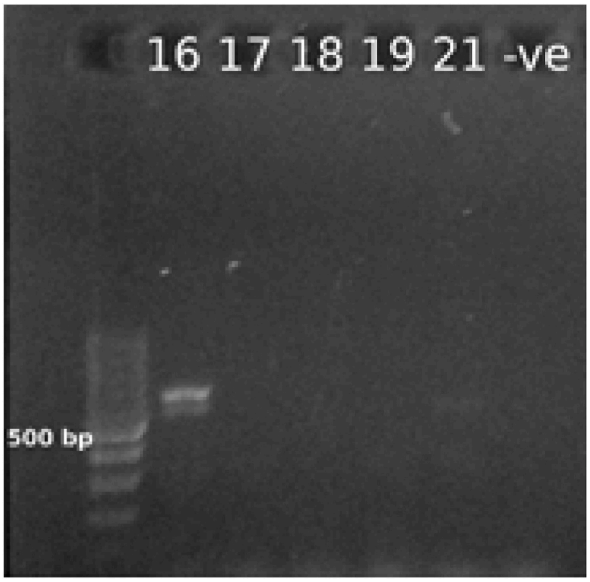

Fig. 3. Gel elecrophoresis of multiplex PCR products of clinical samples in lanes 16–21 with a PCR negative control in the lane marked -ve. Lane 16 indicates positive amplification with two bands larger than 500 bp. Lanes 17–21 indicate no PCR amplification. The sample in lane 16 showed a positive result when tested on the LFA strip. DNA sequencing confirmed the species as *C. glabrata*.

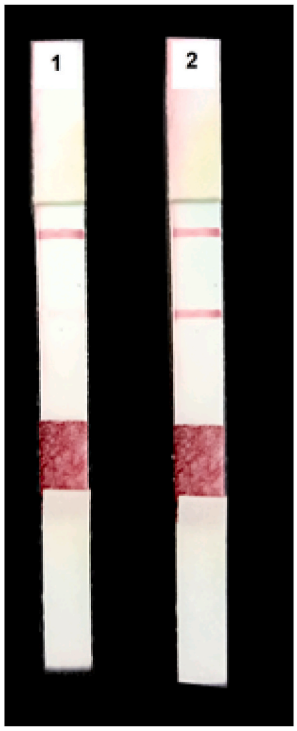

Fig. 4. The developed LFA strips, tested with multiplex PCR products showing the control line only for a negative sample (no. 1) while both control and positive lines are visible for *C. glabrata* positive sample (no. 2).

and clinical requirement for species identification. Furthermore, the PCR can be adapted to detect other fungal species by using appropriate species-specific primers, labelled with digoxygenin for detection via the LFA.

The performance of our PCR-based LFA was mixed with moderately poor sensitivity and excellent specificity. However, the positive and negative predictive value and accuracy are high. This could translate into a promising performance of the PCR-based LFA in

detecting the presence of fungi in clinical samples. Several studies employing similar techniques have reported promising results. High sensitivity and specificity were reported for a rapid PCR-Nucleic Acid Lateral Flow Immunoassay (PCR-NALFIA) method based on rDNA IGS sequence analysis for the detection of a soil fungus, *Macrophomina phaseolina* [7], while a single-tube nested PCR-lateral flow biosensor assay was used for rapid and accurate detection of *Alternaria panax* Whetz, a fungus affecting ginseng [8]. A method with a slight advantage over conventional PCR, as it is conducted isothermally, is recombinant polymerase amplification. It was combined with lateral flow strips for the detection of deep-seated *Candida krusei* infections [9]. Another interesting application of this method is the rapid, and accurate detection of *Cryptococcus* spp. in cerebrospinal fluid with high overall sensitivity and specificity of 95.2 and 95.8 % respectively [10].

Limitations of the techniques used in our study included the need for nucleic acid extraction and amplification before applying the LFA, which we think incurred more time in comparison to directly applying the clinical samples onto the LFA strips. However, these steps were necessary to increase the specificity of the tests.

5. Conclusion

The developed PCR-LFA assay for detection of *Candida* and *Aspergillus* species were tested using spiked samples. The assay recorded 100 % analytical evaluation on fungal-spiked specimens. The clinical evaluation had a high accuracy rate and specificity value. The low sensitivity of this assay can be attributed to the difficulty of extracting fungal DNA from whole blood compared to other bodily fluids. However, the high positive and negative predictive values signal the potential value of this assay as a tool for diagnosing IFDs. Moreover, as an advantage, the turnaround time for the assay is much shorter than conventional fungal culture investigations. Future studies should focus on improving the DNA extraction procedures to improve the sensitivity of the assay.

CRediT authorship contribution statement

Ariff Khalid: Validation, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Jacinta Santhanam:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization. **Mohd Nizam Tzar:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Funding acquisition, Conceptualization. **Ang-Lim Chua:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization. **Sharifah Fadilah Abdul Wahid:** Writing – review & editing, Visualization, Resources, Conceptualization. **Wan Rahiza Wan Mat:** Writing – review & editing, Visualization, Resources, Conceptualization. **Raha Abd Rahman:** Writing – review & editing, Visualization, Resources, Conceptualization. **Petrick Periyasamy:** Writing – review & editing, Visualization, Resources, Conceptualization.

Ethical approval and informed consent statement

Study has been approved by the Universiti Kebangsaan Malaysia Research Ethics Committee (UKM 1.5.3.5/244/JEP-2016-059) and the Medical Research and Ethics Committee at the Ministry of Health Malaysia (NMRR-16-1993-30782). Clinical specimens from patients were collected after obtaining written informed consent from participating patients.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Utility patent filed in 2022 in Malaysia for "Method of Detecting Invasive Fungal Infection, Genus and Species Associated Thereof using Combined Polymerase Chain Reaction Amplification and Lateral Flow Assay" (PI 2022006595).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2025.e42245.

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