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Original Article

Development and clinical implications of a novel CRISPR-based diagnostic test for pulmonary *Aspergillus fumigatus* infection

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KEYWORDS
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Aspergillosis;
Diagnosis;
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Abstract *Background:* Rapid and reliable diagnostic methods for *Aspergillus fumigatus* infection are urgently needed. Clustered regularly interspaced short palindromic repeat (CRISPR)-associated protein 13a (Cas13a) has high sensitivity and specificity in the diagnosis of viral infection. However, its potential use in detecting *A. fumigatus* remains unexplored. A highly sensitive and specific method using the CRISPR/Cas13a system was developed for the reliable and rapid detection of *A. fumigatus*.

Methods: The conserved internal transcribed spacer (ITS) region of *A. fumigatus* was used to design CRISPR-derived RNA (crRNA) and the corresponding recombinase polymerase amplification (RPA) primer sequence with the T7 promoter for the CRISPR assay. Twenty-five clinical isolates and 43 bronchoalveolar lavage fluid (BALF) remaining from routine examinations of patients with confirmed pulmonary aspergillosis were collected to further validate the CRISPR assay.

Results: No amplification signal was observed when genomic DNA from closely clinically related *Aspergillus* species, such as *Aspergillus flavus*, *Aspergillus niger*, and *Aspergillus terreus*, as well as *Monascus purpureus* Went and *Escherichia coli*, was tested by this assay, and the detection limit for *A. fumigatus* was 3 copies in a single reaction system. Validation experiments using the 25 clinical isolates demonstrated 91.7% specificity for the *A. fumigatus* section, and the sensitivity was 100% when first-generation sequencing was used as the standard. There was no significant difference between the PCR and CRISPR methods ($P = 1.0$), and the diagnosis results of the two methods were consistent (Kappa = 0.459, $P = 0.003$).

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Conclusion: The study offers a new validated CRISPR/Cas13a technique for *A. fumigatus* detection, providing a simple, rapid and affordable test that is ready for application in the diagnosis of *A. fumigatus* infection.

Introduction

In China, *Aspergillus fumigatus* is the most commonly isolated *Aspergillus* species (59.3%) in humans and one of more than 30 *Aspergillus* species implicated in invasive aspergillosis (IA).[1] The diagnosis of aspergillosis is challenging. Early diagnosis and species identification are key elements for the treatment of the disease. However, traditional culture methods are time-consuming, and the sensitivity achieved with cultures of lower respiratory tract samples in the diagnosis of invasive pulmonary aspergillosis (IPA) ranges from 5 to 75%, depending on the patient population.[2] The sensitivity of microscopy for diagnosis of IA is 50% at best.[3] The sensitivity of serological tests is influenced by the patient's immune status and the use of antifungal agents.[4] Current nucleic acid detection methods are sensitive and highly adaptable, but most require extensive sample manipulation and expensive machinery. The clustered regularly interspaced short palindromic repeat (CRISPR)—CRISPR-associated protein 13a (Cas13a) technique does not require expensive equipment and reduces the cost and time for diagnosis.[5] Importantly, when combined with a DNA amplification step, this CRISPR system can detect target nucleic acid molecules at a sensitivity as low as 5 aM.[6] The sensitivity and specificity of this system in diagnosis have been demonstrated for Zika virus (ZIKV), *Escherichia coli* and *Pseudomonas aeruginosa*.[7–9] Gootenberg et al. identified *E. coli* and *P. aeruginosa* and differentiated these bacteria from *Mycobacterium tuberculosis*, *Klebsiella pneumoniae* and *Staphylococcus aureus*. Different drug resistance genes of *K. pneumoniae* could be distinguished. Moreover, the CRISPR/13a system can distinguish targets differing in only a single base pair and has been used to successfully distinguish targets such as African and American strains of ZIKV. Recently, CRISPR/Cas13-based SHERLOCK protocols described by Prof. Feng Zhang et al. provided a rapid and accurate diagnostic assay for the novel emerging coronavirus disease 2019 (COVID-19) pneumonia. Therefore, CRISPR/Cas-based technology has good potential for application as a fast and accurate diagnostic assay for infectious diseases.[8] However, there is no relevant information on the diagnosis of *A. fumigatus* infection. Here, we aimed to develop a CRISPR/Cas13a-based rapid diagnostic test for *A. fumigatus*.

Methods

Sample collection

For laboratory validation, we used a standard *A. fumigatus* strain (GIM3.524, Guangdong Microbial Culture Collection Center, China) as an external positive control. For verification of clinical accuracy, we collected 43 samples of residual BALF from patients diagnosed with pulmonary aspergillosis after routine examination and 25 clinical strains from confirmed pulmonary aspergillosis patients in the First Affiliated Hospital of Guangzhou Medical University, including samples of both *A. fumigatus* infection and non-*A. fumigatus* infection, from 1 June 2017 to 30 June 2019. The final clinical diagnosis of pulmonary *A. fumigatus* infection patients was diagnosed according to the Diagnosis and Management of *Aspergillus* Diseases: Executive Summary of the 2017 ESCMID-ECMM-ERS Guideline.[10] The 43 BALF specimens were sent for culture, PCR and metagenomic next-generation sequencing (mNGS). The remaining BALF specimens were stored in a −80 °C freezer before examination by the CRISPR/Cas13a system. PCR assays and mNGS were performed following the kit manufacturer's instructions. Twenty-five clinically isolated *Aspergillus* strains were cultured from BALF from patients with confirmed pulmonary aspergillosis and sent for Sanger sequencing. The exclusion criteria included 1) incomplete clinical data, 2) incomplete microbiological data, and 3) insufficient specimens for CRISPR analysis.

Cas13a protein and other reagents

The primers and plasmids used to clone the target sequence were synthesized by Guangzhou Ige Biotechnology Ltd. (Guangzhou, China). The signal-reporting probe was synthesized by Sangon Co., Ltd. (Shanghai, China). The Cas13a protein used in the study was LwCas13a. LwCas13a was expressed and purified by GenScript (Scotch Plains, NJ). Aliquots of the purified protein were stored at −80 °C. Recombinase polymerase amplification (RPA) enzyme premix (phosphocreatine, 20—80 mM), creatine kinase (50—150 mM), dNTPs (100—300 μM), ATP (20—80 mM), dithiothreitol (DTT; 1—10 mM), potassium acetate (50—200 mM), the recombinant enzymes UxsX (50—300 ng/μL) and UxsY (10—100 ng/μL), single-stranded DNA-binding protein (SSB; 200—1000 ng/μL), and Bsu polymerase (10—100 ng/μL) were purchased from Hangzhou ZC Bio-Sci & Tech Co. Ltd. (Hangzhou, China). Other biochemical reagents from imported or domestic sources were of analytical-grade purity.

Oligos and RNA

We selected the internal transcribed spacer (ITS) region of the *A. fumigatus* genome as the target detection sequence. After sequence alignment and screening, we designed primers and CRISPR-derived RNA (crRNA) that could

specifically detect *A. fumigatus* (CICC®2168). However, they did not cross-react with other fungal strains, such as *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus oryzae*, and *Aspergillus terreus* (China Center of Industrial Culture Collection; CICC®2004, CICC®2039, CICC®2001 and CICC®2425, respectively), and had a high degree of sensitivity. Primer sets, including the forward primer 5′-TAATACGACTCACTATAGGGGGTCCAACCTCCC ACCCGTGTCTATC-3′ and reverse primer 5′- TCGATGATTCACTGAATTCTGC AATTCACATTAC-3′, were synthesized for amplification of the ITS region for *A. fumigatus* detection. The *A. fumigatus* crRNA 5′-GGGGAUUU AGACUACCCCAAAAACGAAGGGGACUAAAACUUACGAUAAUCAACUCAGACUGCAUACU-3' (SEQ ID NO: 1) was used in this study.

CRISPR assay

The DNA from the BALF samples was extracted with a commercial extraction kit (DNeasy Plant Mini Kit, Qiagen, Germany) according to the manufacturer's recommendations. The CRISPR test combined an RPA step and a subsequent Cas13a detection step. Briefly, 10 μM forward and reverse primers were mixed in 21 μL of RPA mix, and the solution was centrifuged, after which, 1 μL of LwCas13a, 1 μL of crRNA, 0.2—2 μL of T7 RNA polymerase mix, 1—10 μL of NTP mix, and 1 μL of the signal-reporting probe were added. This final reaction was incubated at 37 °C and monitored for a fluorescence signal for 30 min. Fluorescence signals were measured by an ABI7500 instrument (Thermo Fisher, Massachusetts) for a duration of 30 min. FAM fluorescence values were read every 1 min. The positive criterion was a fold change value > 3.0.

PCR assay

A commercially available multiplex real-time PCR kit (AsperGenius®, PathoNostics, Maastricht, the Netherlands) was used according to the manufacturer's recommendations.[11] DNA was extracted from the supernatant by using NucliSENS® EasyMAG from bioMérieux (France; Marcy l'Etoile). The AsperGenius® multiplex assays were validated on real-time LightCycler 480 II instruments. A sample was defined as positive by a Ct $\leq$ 38.

mNGS assay

The extracted DNA was used to construct NGS libraries through DNA fragmentation, end repair, adaptor ligation and PCR amplification. Agilent 2100 and Qubit 3.0 instruments were used for qualification and quantification of the DNA libraries. Qualified libraries were then sequenced on a NextSeq CN500 for 75 cycles. Raw sequencing data were processed with fastp to remove low-quality and short (length $<$ 35 bp) reads. Host reads were then mapped to the human reference genome (hg19) using Burrows-Wheeler Alignment with default parameters.[12] Low-complexity reads were removed by Kcomplexity with default parameters.[13] Clean reads were mapped against the National Center for Biotechnology Information nucleotide database (version 1, July 2019) using BLAST + software (version 2.9.0) (-task megablast, -evalue 1 × 10—5, -max_target_seqs 10, -max_hsps 1).[14] Taxonomic assignment was performed with MEGAN software (version 6.11.0) using the lowest-common-ancestor algorithm (-ms 100, -supp 0, -me 0.01, -top 10, -mrc 60).[15] When the fungal reads per million (rpm) value of the sample was 10-fold higher than that of the negative control, we considered the sample to be positive for the pathogen. A homemade script was used to extract the taxonomic abundance for further analysis.

Sanger sequencing

cDNA (2 μL) was amplified using Phoenix™ Hot Start Taq DNA Polymerase (Enzymatics) in a 20-μL PCR mix containing 4 μL of 5X Phoenix Hot Start Taq Reaction Buffer, 2 μL of dNTPs (2.5 μM), 0.5 μL each of forward and reverse primers (10 μM), 0.2 μL of Phoenix™ Hot Start Taq DNA Polymerase (500 U), 1 μL of template DNA (10 ng), and 12.3 μL of nuclease-free water (not DEPC-treated). The PCR products were visualized via agarose gel electrophoresis, cleaned up using AMPure XP beads (Beckman Coulter, Miami, FL, USA) and then subjected to sequencing reactions using either the M13F or M13R primer with Big Dye Terminator Reaction Mix (Applied Biosystems). The reaction products were purified by the Big Dye XTerminator Purification Kit (Applied Biosystems) and examined on an ABI 3500 XL sequencer.

Identification of aspergillus isolates based on morphological and cultural characteristics

The clinical isolates (n = 25) comprised the common pathogenic *Aspergillus* species. All the fungal samples submitted to the Clinical Microbiology Laboratory were routinely plated on Sabouraud dextrose agar plates with chloramphenicol and gentamicin. The plates were incubated at 35 °C with 5% CO_2 for a total of 1 week, and observations were recorded twice per week. *A. fumigatus* was observed under a microscope after staining with 10% KOH and lactic acid Medan. *Aspergillus* species were identified using standard morphological criteria.

Data processing and statistical analysis

DEPC-treated water purchased from Sangon Biotech (Shanghai China) was used as a negative control (NC). The samples yielding a 3.0-fold or greater fluorescent signal over the NC were determined to be positive. The chi-square test was employed to evaluate binomial variables. Statistical analyses and figure construction were conducted using SPSS Statistics 25.0 software and GraphPad Prism 5 software.

Results

Development of the CRISPR assay

Based on the principle underlying the CRISPR/Cas13a system, the target sequence was isothermally amplified through RPA technology. Then, using T7-mediated *in vitro* transcription, the DNA sequence was transcribed to the

target RNA that could be directly bound by Cas13a. Under the guidance of crRNA, Cas13a recognizes and binds to target RNA, and at the same time, its collateral cleavage activity is activated to cut the reporter RNA molecule, with fluorescent groups added earlier in the reaction system being released to yield fluorescent signals indicating the presence of the target nucleic acid. We developed a rapid, highly sensitive and simple assay by combining an RPA reaction with T7 transcription to convert amplified DNA to RNA for subsequent detection by LwCas13a (Fig. 1).

To establish a rapid and simple CRISPR assay, we set out to screen the crRNA and RPA primers. First, 2 crRNAs were designed for the target sequence for optimization and screening. Based on these 2 crRNA sequences, two single-stranded sequences containing the T7 promoter with specific recognition sites were synthesized as templates (Supplementary Table 1), thereby preventing the crRNA signal from being affected due to the difference in the amplification primers. An ABI7500 fluorescence detector was used to detect and screen the crRNA of *A. fumigatus*, and the results are shown in Fig. 2(a). The crRNA-1 signals were all ideal.

Multiple pairs of RPA primers were designed and optimized for the *A. fumigatus* ITS region. The designed primer amplification products ranged in length from 110 to 260 bp, and 4 forward primers and 4 reverse primers (Supplementary Table 2) were designed for the ITS region. There was no statistically significant difference between the fold changes obtained with F4R1 and F4R3. The reproducibility of F4R1 was better than that of F4R3 (Wilcoxon rank sum test, p-value = 1.083e-05). The primer pair F4R1 was finally selected by using an ABI7500 fluorescence detector, as shown in Fig. 2(b).

To determine the analytical sensitivity of the CRISPR/Cas13a assay, a plasmid with a conserved sequence of *A. fumigatus* was used as a template, and 5 concentrations, namely, 3000 copies/L, 300 copies/L, 30 copies/L, 3 copies/L, and 0 copies/L, were used for evaluation of the limit of detection (LOD). The results showed that the *A. fumigatus* detection limit was 3 copies in a single reaction system, as shown in Fig. 2(c).

The specificity of the CRISPR system for *A. fumigatus* was verified based on RPA combined with T7 transcription and Cas13a. *A. fumigatus* genomic DNA, plasmid DNA with the target sequence, and genomic DNA from *A. flavus*, *A. niger*, *A. terreus*, *Aspergillus nidulans*, *Aspergillus tamarii*, *Monascus purpureus* Went, *Penicillium citrinum*, *Streptococcus agalactiae*, *M. tuberculosis*, and *E. coli* were used to evaluate specificity. Samples of the genome were diluted to 10^4 copies/L. HeLa cell genomic DNA was used as a negative quality control. As shown in Fig. 2(d), only *A. fumigatus* genomic DNA and plasmid DNA containing the target sequence could be specifically detected.

Application of the CRISPR assay for clinical *A. fumigatus* strains

After establishing the CRISPR assay, we set out to evaluate its efficacy with clinical strains. As shown in Fig. 3, we isolated 25 native *Aspergillus* isolates from confirmed pulmonary aspergillosis patients, and the isolates were all identified by culture, Sanger sequencing and the CRISPR assay. The Sanger sequencing results identified the following strains: 13 *A. fumigatus*, 1 *A. niger*, 1 *Aspergillus ustus*, 1 *Talaromyces wortmannii*, 1 *Aspergillus sydowii*, 1 *Monascus anka*, 2 *A. tamarii*, 1 *A. nidulans*, 3 *A. flavus*, and 1 *P. citrinum* strain. The thirteen strains of *A. fumigatus* identified by Sanger sequencing were also positively identified as *A. fumigatus* by the CRISPR assay and culture methods. The CRISPR assay identified 1 of the 12 non-*A. fumigatus* strains (as determined by Sanger sequencing) as *A. fumigatus*, while the culture method identified 5 of these strains as *A. fumigatus*. The sensitivity and specificity of the CRISPR method were 100% and 91.7%, respectively, while those of the culture method were 100% and 58.3%, respectively.

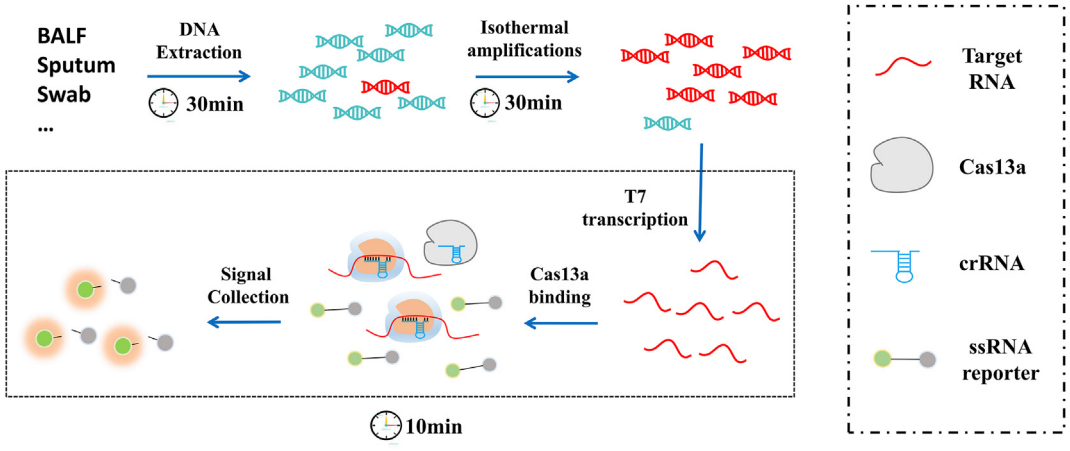

Figure 1. Schematic diagram of the CRISPR-*A. fumigatus* assay. Samples were first eluted, followed by a rapid DNA extraction step where the cell walls were disrupted by a combination of chemical, physical and heating effects. The extracted DNA was then subjected to the CRISPR/Cas reaction. The collateral nuclease activity of the Cas proteins was activated upon specific binding of crRNA to the *A. fumigatus* gene. The fluorescent signal produced from the cleaved probes was captured and indicated the presence of *A. fumigatus*.

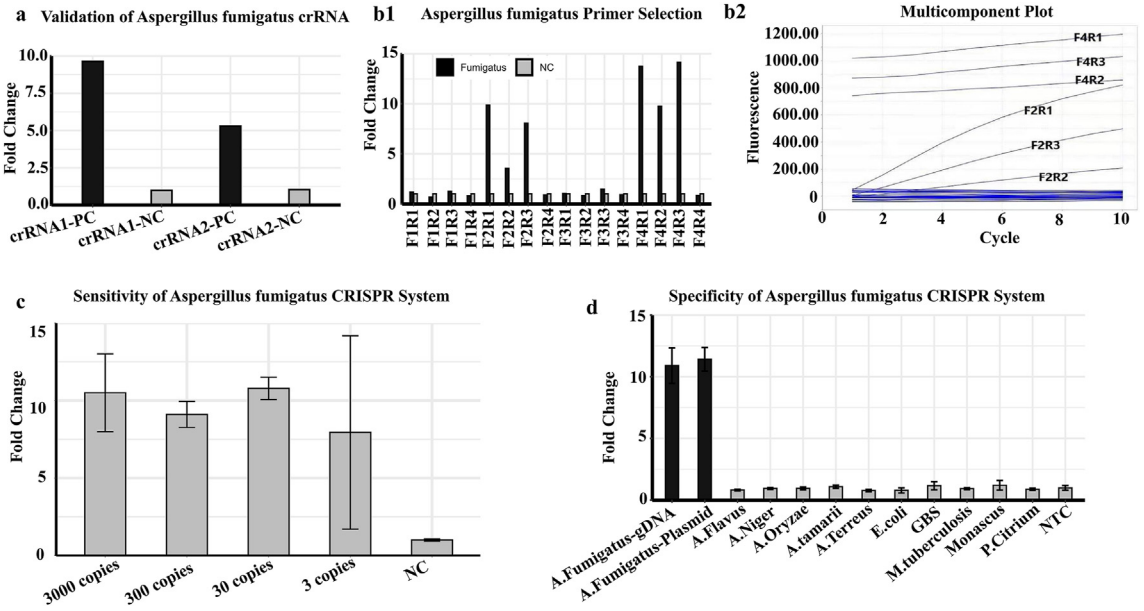

Figure 2. Development of the CRISPR assay. (a) The signal of crRNA-1 was better than that of crRNA-2. (b1) There was no statistically significant difference between the fold change obtained with F4R1 and F4R3. Black bars represent positive results with the DNA template in the reaction system, and gray bars represent the negative control without the DNA template. (b2) The reproducibility of F4R1 was better than that of F4R3. All tests were performed in ten replicates. (c) The detection limit for *Aspergillus fumigatus* was 3 copies per reaction system. (d) Only genomic DNA of *Aspergillus fumigatus* and plasmid DNA containing the target sequence could be detected specifically, and the other samples showed no obvious signal.

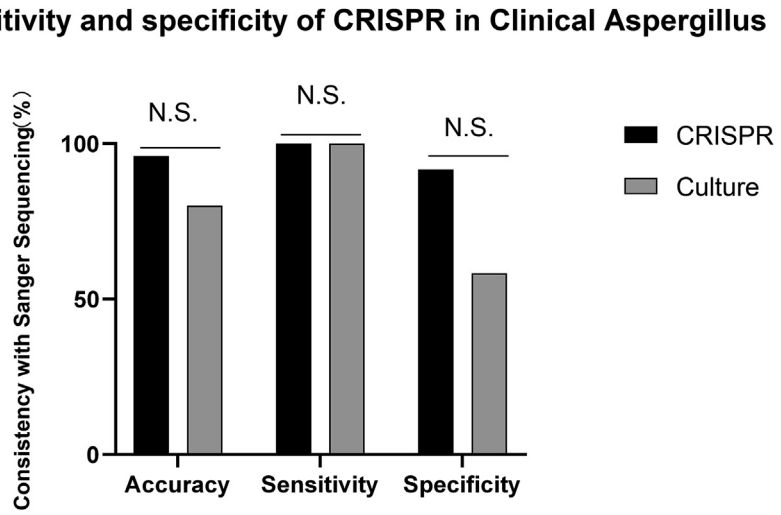

Figure 3. Evaluation of the sensitivity and specificity of CRISPR in the detection of clinical *Aspergillus* strains. The sensitivity and specificity of the CRISPR method were 100% and 91.7%, respectively, while those of the culture method were 100% and 58.3%, respectively, with Sanger sequencing used as the standard.

Application of the CRISPR assay for BALF from patients with confirmed pulmonary aspergillosis

As shown in Table 1, compared with the *A. fumigatus* mNGS method, CRISPR had a diagnostic sensitivity of 55.6% and a specificity of 93.8%. The sensitivity and specificity of the culture method were 7.4% and 100%, respectively, and the sensitivity and specificity of the PCR method were 51.9% and 81.3%, respectively. There was no significant difference between the PCR and CRISPR methods (P = 1.0), and the results of the two methods were consistent (Kappa = 0.459, P = 0.003).

Discussion

Unlike Cas9, the RNA-guided and RNA-targeting CRISPR effector Cas13a exhibits a "collateral effect" of promiscuous RNAse activity upon target recognition. We combined

Table 1 Examination results for patients with confirmed pulmonary aspergillosis.

Patient	Age	Sex	GM	IgG	IgM	Diagnosis	Specimen	Culture	CRISPR	mNGS	PCR
1	22	F	+	+	+	pathology	BALF	AF	−	AF	−
2	56	F	±	−	−	pathology	BALF	un	−	Other	−
3	47	F	+	−	−	pathology	BALF	un	AF	Other	−
4	53	M	±	+	−	pathology	BALF	AF	−	AF	−
5	50	M	+	−	−	pathology	BALF	un	−	Other	−
6	41	M	±	±	−	pathology	BALF	un	−	Other	−
7	45	M	+	±	−	pathology	BALF	un	AF	AF	AF
8	51	F	±	+	+	pathology	BALF	un	AF	AF	AF
9	43	F	±	+	−	pathology	BALF	un	−	Other	−
10	51	M	±	±	+	pathology	BALF	un	−	Other	AF
11	63	M	−	−	−	pathology	BALF	Other	−	Other	AF
12	57	M	−	±	+	pathology	BALF	un	AF	AF	−
13	61	M	−	±	−	pathology	BALF	un	−	AF	AF
14	53	M	−	−	−	pathology	BALF	un	−	Other	−
15	68	M	−	±	+	pathology	BALF	un	−	AF	−
16	55	M	+	+	−	pathology	BALF	un	−	Other	−
17	58	M	+	−	+	pathology	BALF	un	−	Other	AF
18	61	M	+	−	−	pathology	BALF	un	AF	AF	AF
19	57	M	−	−	−	pathology	BALF	un	AF	AF	AF
20	37	F	+	+	−	pathology	BALF	un	−	Other	−
21	46	M	+	+	−	pathology	BALF	Other	−	AF	−
22	57	M	−	−	−	pathology	BALF	un	AF	AF	−
23	58	M	−	−	−	pathology	BALF	un	−	AF	AF
24	28	M	+	±	−	pathology	BALF	un	−	AF	AF
25	45	M	±	−	−	pathology	BALF	un	−	AF	−
26	72	M	−	+	−	pathology	BALF	un	−	AF	−
27	49	M	−	±	−	pathology	BALF	un	AF	AF	AF
28	25	F	+	−	−	pathology	BALF	un	−	AF	−
29	61	M	−	±	−	pathology	BALF	un	AF	AF	AF
30	21	M	−	−	±	pathology	BALF	un	−	AF	−
31	50	M	+	−	−	pathology	BALF	un	−	Other	−
32	72	M	−	+	−	pathology	BALF	un	AF	AF	−
33	39	M	+	−	−	pathology	BALF	un	−	Other	−
34	51	F	±	+	+	pathology	BALF	un	AF	AF	AF
35	48	F	−	+	−	pathology	BALF	un	AF	AF	AF
36	60	M	+	−	−	pathology	BALF	un	AF	AF	AF
37	32	M	−	±	−	pathology	BALF	un	AF	AF	−
38	57	M	+	−	−	pathology	BALF	un	−	Other	−
39	67	M	−	+	+	pathology	BALF	un	AF	AF	AF
40	40	M	±	−	−	pathology	BALF	un	−	Other	−
41	38	F	+	−	−	pathology	BALF	un	−	AF	−
42	58	M	+	−	+	pathology	BALF	un	AF	AF	AF
43	43	M	−	+	−	pathology	BALF	un	−	Other	−

F: female; M: male; -: negative; ±: weakly positive; +: positive; un: unknown; AF: *Aspergillus fumigatus*; other: non-*Aspergillus fumigatus*; GM: galactomannan.

the collateral effect of Cas13a with isothermal amplification to establish a CRISPR-based diagnostic assay providing rapid DNA or RNA detection with high sensitivity and specificity.[7] There was no statistically significant difference in sensitivity or specificity between the CRISPR and PCR methods in this study. To the best of our knowledge, this is the first study that aimed to establish a CRISPR/Cas13a detection system for *A. fumigatus* and to further verify the results with strains and clinical specimens. In terms of the diagnostic procedure, the RPA method eliminates the complexity introduced by variable temperatures. Its overall turnaround time is shorter than that of PCR, which makes it a promising alternative for the rapid diagnosis of *A. fumigatus* infection.

Multiple genes, ranging from the universal ITS ribosomal DNA regions and the large ribosomal subunit D1-D2 to protein-coding genes, such as the β-tubulin and calmodulin gene regions, have been evaluated to delimit species within aspergilli.[16] The ITS region is the most extensively used nuclear ribosomal gene region for specific identification and phylogenetic analysis of fungal groups and has been declared the DNA barcode for fungi.[17] The ITS region

contains variable elements that allow sequence-based identification of *Aspergillus* species.[18] Koichi et al. developed a PCR system in which a primer pair was designed from conserved sequences of ITS1 ribosomal DNA and its flanking regions that allowed recognition of three major pathogenic *Aspergillus* species, namely, *A. fumigatus*, *A. niger* and *A. flavus*.[19] Previous studies have shown that the sensitivity and specificity of PCR of BALF from rabbits with experimental IPA caused by *A. fumigatus* species are 100% and 96%, respectively, before treatment and 39% and 44%, respectively, after treatment.[20] This is basically consistent with the results of this study. This study showed that the CRISPR system had a diagnostic sensitivity of 55.6% and a specificity of 93.8% in BALF samples, with mNGS results as the standard. PCR had a sensitivity and specificity of 51.9% and 81.3%, respectively. The sensitivity and specificity of diagnosis by the CRISPR system in clinical strains were 100% and 91.7%, respectively, with the first-generation sequencing results as the standard, and one case was identified as a false positive. The CRISPR system showed high sensitivity and specificity in the diagnosis of *A. fumigatus* strains.

In some BALF samples, mNGS gave positive results, while PCR gave negative results. Sequence-based methods are susceptible to nucleotide substitution, which affects oligonucleotide hybridization efficiency and results in false negatives.[21] The CRISPR assay also gave false negatives. This condition may be related to off-target effects,[22,23] which means that Cas nucleases exhibit off-target activity on sequences similar to the intended target. Other factors can also affect the results of the CRISPR assay, such as antifungal drugs. Non-*A. fumigatus* strains may also produce positive results due to interspecies sequence similarity within *Aspergillus*; thus, the CRISPR assay has the potential to yield false positives. Regarding methodology, the mNGS, PCR and CRISPR methods are significantly different. The PCR and CRISPR methods mainly target a single gene. However, mNGS, in addition to the targets of the CRISPR assay, also covers other areas of the *A. fumigatus* genome, which can prevent false negatives caused by mutations in the CRISPR target area and help researchers obtain more sequence information and assemble whole-genome sequence information for tracing the source for evolutionary analysis, discovery and analysis of mutations.

The diagnostic efficacy of the AsperGenius® PCR assay has been demonstrated.[11] This study confirmed the consistency of the results of the CRISPR and PCR methods. Generally, the CRISPR method, due to its rapid detection, simple operation, low cost, and diagnostic efficiency consistent with that of PCR, is more suitable for screening to rapidly obtain preliminary evidence of *Aspergillus* infection. For patients who are CRISPR-negative but have a clinical phenotype that is highly suspected to be due to *Aspergillus*, further confirmation using mNGS can effectively improve the reliability of test results. In addition, for samples that test positive by the CRISPR detection method, mNGS can also be further used to complete sequence analysis to help obtain information on drug-resistant strains.

There are some limitations to this study. First, our study was limited by a small sample size, and some false positives and false negatives may not have been observed. Second, ITS regions may not provide sufficient sensitivity for discriminating among individual species within a section.[16] *A. fumigatus* belongs to the section *Fumigati*, in which 63 species have now been described. While resistance to triazoles is rare among *A. fumigatus sensu stricto*, these sibling or cryptic species commonly exhibit decreased susceptibility to azoles and other antifungal agents.[16] Considering that definitive species identification requires specific sequencing analyses of the beta-tubulin or calmodulin genes, which are fastidious and not convenient for rapid identification in clinical practice, and that not all species are pathogenic, further identification of sibling or cryptic species is required according to the clinician's needs.

The current study presents for the first time a CRISPR-based method for *A. fumigatus* infection diagnosis. The sensitivity and specificity of the CRISPR effector Cas13a for *A. fumigatus* detection were consistent with those of PCR, and the assay was quick, sensitive, and economical. It has high potential for application in the diagnosis of *A. fumigatus* infection.

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References

1. Chen M, Xu Y, Hong N, Yang Y, Lei W, Du L, et al. Epidemiology of fungal infections in China. *Front Med* 2018;**12**:58—75.
2. Soubani AO, Khanchandani G, Ahmed HP. Clinical significance of lower respiratory tract Aspergillus culture in elderly hospitalized patients. *Eur J Clin Microbiol Infect Dis* 2004;**23**:491—4.
3. Ruchel R, Schaffrinski M. Versatile fluorescent staining of fungi in clinical specimens by using the optical brightener Blankophor. *J Clin Microbiol* 1999;**37**:2694—6.
4. Richardson MD, Page ID. Aspergillus serology: have we arrived yet? *Med Mycol* 2017;**55**:48—55.
5. Hou T, Zeng W, Yang M, Chen W, Ren L, Ai J, et al. Development and evaluation of a rapid CRISPR-based diagnostic for COVID-19. *PLoS Pathog* 2020;**16**:e1008705.
6. Abudayyeh OO, Gootenberg JS, Konermann S, Joung J, Slaymaker IM, Cox DB, et al. C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. *Science* 2016;**353**:f5573.
7. Gootenberg JS, Abudayyeh OO, Lee JW, Essletzbichler P, Dy AJ, Joung J, et al. Nucleic acid detection with CRISPR-Cas13a/C2c2. *Science* 2017;**356**:438—42.
8. Xiang X, Qian K, Zhang Z, Lin F, Xie Y, Liu Y, et al. CRISPR-cas systems based molecular diagnostic tool for infectious diseases and emerging 2019 novel coronavirus (COVID-19) pneumonia. *J Drug Target* 2020;**28**:727—31.
9. Liu Y, Xu H, Liu C, Peng L, Khan H, Cui L, et al. CRISPR-Cas13a nanomachine based simple technology for avian influenza A (H7N9) virus on-site detection. *J Biomed Nanotechnol* 2019;**15**:790—8.
10. Ullmann AJ, Aguado JM, Arikan-Akdagli S, Denning DW, Groll AH, Lagrou K, et al. Diagnosis and management of Aspergillus diseases: executive summary of the 2017 ESCMID-ECMM-ERS guideline. *Clin Microbiol Infect* 2018;**24**(Suppl 1):e1—38.
11. Pelzer BW, Seufert R, Koldehoff M, Liebregts T, Schmidt D, Buer J, et al. Performance of the AsperGenius(R) PCR assay for

detecting azole resistant Aspergillus fumigatus in BAL fluids from allogeneic HSCT recipients: a prospective cohort study from Essen, West Germany. *Med Mycol* 2020;**58**:268—71.
12. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* 2009;**25**:1754—60.
13. Clarke EL, Taylor LJ, Zhao C, Connell A, Lee JJ, Fett B, et al. Sunbeam: an extensible pipeline for analyzing metagenomic sequencing experiments. *Microbiome* 2019;**7**:46.
14. Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, et al. BLAST+: architecture and applications. *BMC Bioinf* 2009;**10**:421.
15. Huson DH, Beier S, Flade I, Gorska A, El-Hadidi M, Mitra S, et al. MEGAN community edition - interactive exploration and analysis of large-scale microbiome sequencing data. *PLoS Comput Biol* 2016;**12**:e1004957.
16. Balajee SA, Houbraken J, Verweij PE, Hong SB, Yaghuchi T, Varga J, et al. Aspergillus species identification in the clinical setting. *Stud Mycol* 2007;**59**:39—46.
17. Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, et al. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proc Natl Acad Sci U S A* 2012;**109**:6241—6.
18. Iwen PC, Hinrichs SH, Rupp ME. Utilization of the internal transcribed spacer regions as molecular targets to detect and identify human fungal pathogens. *Med Mycol* 2002;**40**:87—109.
19. Sugita C, Makimura K, Uchida K, Yamaguchi H, Nagai A. PCR identification system for the genus Aspergillus and three major pathogenic species: Aspergillus fumigatus, Aspergillus flavus and Aspergillus Niger. *Med Mycol* 2004;**42**:433—7.
20. Walsh TJ, Wissel MC, Grantham KJ, Petraitiene R, Petraitis V, Kasai M, et al. Molecular detection and species-specific identification of medically important Aspergillus species by real-time PCR in experimental invasive pulmonary aspergillosis. *J Clin Microbiol* 2011;**49**:4150—7.
21. Alvarez-Diaz DA, Franco-Munoz C, Laiton-Donato K, Usme-Ciro JA, Franco-Sierra ND, Florez-Sanchez AC, et al. Molecular analysis of several in-house rRT-PCR protocols for SARS-CoV-2 detection in the context of genetic variability of the virus in Colombia. *Infect Genet Evol* 2020;**84**:104390.
22. Klein M, Eslami-Mossallam B, Arroyo DG, Depken M. Hybridization kinetics explains CRISPR-cas off-targeting rules. *Cell Rep* 2018;**22**:1413—23.
23. Jackson AL, Bartz SR, Schelter J, Kobayashi SV, Burchard J, Mao M, et al. Expression profiling reveals off-target gene regulation by RNAi. *Nat Biotechnol* 2003;**21**:635—7.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2021.11.008.