Development and Validation of LAMP Primer Sets for Rapid and Correct Identification of *Aspergillus fumigatus* Carrying the *cyp51A* TR₄₆ Azole Resistance Gene

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ABSTRACT

Infections due to triazole resistant *Aspergillus fumigatus* are increasingly reported worldwide and are associated with treatment failure and mortality. The principal class of azole resistant isolates is characterized by the presence of tandem repeats of 34 bp or 46 bp (TR₃₄ or TR₄₆) within the promoter region of the *cyp51A* gene. Loop-mediated isothermal amplification (LAMP) is a widely used nucleic acid amplification system with high rapidity and specificity. In this paper, we report a new LAMP assay method to detect the 46 bp tandem repeat insertion in the *cyp51A* gene promoter

region, named TR₄₆-LAMP assay, based on the use of a newly designed specific LAMP primer sets. TR₄₆ is a high-prevalence allele which is associated with the occurrence of multi-triazole resistance of *A. fumigatus* in patients as well as isolates from the environment. This newly designed TR₄₆-LAMP assay was validated as a useful method for specific detection of azole-resistant *A. fumigatus* isolates bearing TR₄₆² as well as TR₄₆³ in *cyp51A* gene promoter region. It could also differentiate azole-resistant isolates of TR₄₆ tandem repeats from those with TR₃₄ tandem repeats in *cyp51A* genes. These results showed this TR₄₆-LAMP method is specific, rapid, and also provides crucial insights to enable development of novel antifungal therapeutic strategies against severe fungal infections due to *A. fumigatus* with TR₄₆ tandem repeats.

KEYWORDS: rapid and correct identification, new LAMP assay method, azole resistance, *cyp51A* gene, *Aspergillus fumigatus*

INTRODUCTION

Antimicrobial resistance was defined by the World Health Organization (WHO) as one of the most important threats to human health, since it can compromise our ability to treat infectious diseases, as well as undermining other advances in health care (1). Although bacterial resistance still remains the most common finding in the clinical setting, fungal resistance, specially to azole antifungals among filamentous fungi, is relentlessly increasing worldwide (2,3,4). Resistance to azole drugs can be due, in general, to two major genetic mechanisms; point mutation(s) in *cyp51A* open reading frame with or without tandem repeat (TR) of 34 or 46 base pair (bp) in the promoter region of the gene (TR₃₄ or TR₄₆) and overexpression of oligonucleotide sequence in the *cyp51A* gene (5). These mutations and overexpression of the gene are conferring different levels of resistance (6). As a general concept, the point mutation is the consequence of previous exposure to azole drugs in the clinical setting, such as

for prophylactic approaches or for therapeutic purpose (7,8). On the other hand, the TR with point mutation(s) is understood as the aftermath of previous exposure to azole fungicides. In an agricultural setting, azole fungicides are widely used to prevent fungal contamination in a large variety of crop and plant protection, which may allow the TR type resistant strains to be emerged in the environments and the conidia to disperse to the air (8,9). In Brazil, agrobusiness represents about 25% of the Brazilian Gross National Product (GNP) (<http://www.agricultura.gov.br>), and in this scenario, fungicides use is steadily increasing over the years. In 2010, the consumption of pesticides in Brazil increased 190% in the year and fungicides corresponded to 14% in this market (<https://www.ipessp.edu.br>).

In the Netherlands, prevalence of TR type resistant strains were seriously high. A set of 952 clinical *A. fumigatus* strains were collected and found to include 225 and 98 strains with TR₃₄ and TR₄₆, respectively (10). Another study revealed that 13 TR₃₄ strains and 3 TR₄₆ strains were isolated among 105 clinical strains. Accordingly, there are number of reports assessing fatal case of invasive aspergillosis caused by *A. fumigatus* carrying the TR₄₆ in an acute myeloid leukemia (AML) patient and hematopoietic stem cell transplant recipients (11). Higher mortality of the patients with invasive aspergillosis caused by azole resistant strain has been reported (11,12). Thus, a rapid and specific method which is able to identify the presence of TR would contribute to more timely therapeutic decision making (13).

As one of the promising diagnostic tools for the azole resistant *A. fumigatus*, the high potential of loop-mediated isothermal amplification (LAMP) for the development of improved DNA-based diagnostic kits has been reported (14). In general, the LAMP method was found to be similar or superior to the general PCR method, and more specific, low-cost and simple. Actually LAMP based approaches have been applied to a wide range of samples, such as whole blood, paraffin-embedded tissues, and various microbial pathogens. In this paper, we report a novel LAMP assay method which selectively detects triazole resistant *A. fumigatus* strains due to presence of double or triple TR₄₆ (TR₄₆³) in

cyp51A promoter region.

RESULTS

Antifungal susceptibility tests

Drug susceptibilities of 41 *A. fumigatus* strains against azole drugs itraconazole and voriconazole are shown in Table 2. Thirty strains designated as wild type, were isolates from clinical specimens, and they were confirmed to belong to drug susceptible to itraconazole and voriconazole. Remaining 11 strains were in the azole resistant group, and most of them showed MIC values of $>8 \mu\text{g/ml}$ against voriconazole.

Primer design

The most important step in LAMP assay is the design of primers. In LAMP assay, 6 primers are necessary to amplify the targeted region under isothermal condition. First, we inspected the promoter region (-461 bp to -296 bp counted from start codon) of *cyp51A* gene to select a set of primer sequences that specifically amplify the repeated 46 bp sequence in strains with TR₄₆ mutation (Fig. 1). To enable for specific amplification against repeated TR₄₆ sequences, B2 was set on the joint with two 46 bp sequences. To obtain a specific and rapid LAMP primer set in the LAMP assay, other 5 sequences for primer sets were chosen in the target region according to the standard criteria. Namely 6 primers (F1, F2, F3, B1, B2, B3) that target 6 specific regions of a DNA template of the TR₄₆ gene of *cyp51A* were selected, and in addition 2 loop primers (LF, LB) were also chosen to accelerate the reaction. Based on the above information, several new candidates of LAMP primers were designed and their utility was tested. From those, one useful LAMP primer sets based on the detection of TR₄₆ regions in *cyp51A* gene was selected (Table 1).

Verification of LAMP for TR₄₆

Specificity of the primer sets was tested using various types of *A. fumigatus* strains, such as reference

wild isolates, and environmental or clinical azole resistance isolates (Fig. 2).

As shown in Fig. 3, the start of the LAMP amplification was at around 50 min in the *A. fumigatus* strains (IFM 63432) harboring the TR₄₆ resistant mutation (TR₄₆/Y121F/T289A). In the present study, IFM 63432 strain was used as a positive control one for this LAMP amplification experiment. This TR₄₆ LAMP primer could not amplify the DNA from 30 strains of azole drug susceptible wild type and clinical isolates of *A. fumigatus* (Fig. 3 A-i). As shown in Fig. 3A-ii, TR₄₆ LAMP primer could amplify the DNA from *A. fumigatus* strains carrying the duplicate 46 bp promoter repeat in *cyp51A* gene (IFM63432, Be1-2, BE1-4, BE3-5, BE3-6). When the present TR₄₆ LAMP primer was applied for three strains with TR₃₄ (Table2), TR₃₄/L98H (IFM64460 and 64733) and TR₃₄/L98H/Y289/T289A/I364V/G448S (3-1-B), DNA amplification was not observed. This suggests that the present LAMP primer could not detect TR₃₄ drug resistant strains regardless of their point mutation site in *cyp51A* gene (Fig. 3 A-iii and B-ii). It was also confirmed that TR₄₆ LAMP primer could amplify DNA of *A. fumigatus* strains carrying a TR₄₆³ (TR₄₆³/Y121F/M172I/T289A/G448S) in the *cyp51A* gene (Fig.3A-iii,3B-ii). Therefore, present studies confirmed that newly established TR₄₆ LAMP primer set was only specific *A. fumigatus* strains with TR of double or triple 46-bp promoter tandem repeats in *cyp51A* gene. Colony LAMP verified for 2 strains each carrying TR₄₆, TR₄₆³, and TR₃₄.

DISCUSSION

Azole antifungals mainly inhibit ergosterol biosynthetic pathway by targeting the cytochrome P450-dependent enzyme lanosterol 14- α -demethylase, encoded by *cyp51A* in molds. Resistance to this class of drugs in the major human pathogen *A. fumigatus* is emerging and reaching levels to prevent their clinical use (6). Advances in recent molecular genetic technologies such as real-time PCR have introduced various useful diagnostic assay methods into the fields in azole resistant mechanism analysis. Even in comparison with those of new molecular biological techniques, the LAMP assay

described here has advantages of high sensitivity and specificity, and also low costs and short amplification time. In addition, there have been no reports using LAMP techniques to study azole resistant mechanisms in *A. fumigatus* by TR₄₆ gene. Quite recently Yu Shan-Ling *et al.* (15) reported a successful LAMP technique to detect azole resistant strains due to amplification of a TR of a 34 bp (TR₃₄) within the promoter region of *cyp51A* of *A. fumigatus*. However, their method could not detect azole resistant *A. fumigatus* strains with amplification of a 46 bp of promoter regions. There is a difference in MIC between strains with TR₄₆ and strains with TR₃₄ (16). Thus, the importance of detecting TR₄₆ lies in the fact that strains of *A. fumigatus* harbouring TR₄₆ are resistant to voriconazole, but not to itraconazole, while strains with TR₃₄ are resistant to both drugs (16). In this study, we were succeeded in the design of useful TR₄₆ LAMP primer sets to detect specifically a TR₄₆ within the promoter regions of azole resistant *A. fumigatus*. The designed primer sets could differentiate azole resistant TR₄₆ strains from that of TR₃₄ strains. To our knowledge, this is the first report of a detection method for one of the most prevalent *cyp51A* resistant gene TR₄₆ in *A. fumigatus* azole resistant strains.

Recently, the strain consisting of the triple repeat of 46 bp of the promoter region was reported in the Netherlands (TR₄₆³) (17). The LAMP primer we designed was able to detect both two copies of the TR₄₆ tandem repeat and three copies of the TR₄₆. Moreover, these amplification curves (as well as the starting point) were similar. The BIP (B1 + B2 complementary; Fig. 1) of the primer we designed is TR-specific. B1 is designed at the boundary where the repeat unit is inserted, and B2 is designed at the boundary between the repeat units. In addition, B3 is designed on a repeat unit. Based on the results of strains having double repeat and triple repeat, it was suggested that the primer used this time may be able to detect even if the number of repeats increases.

It is widely known that exposure to azole fungicides resulted in the emergence of azole-resistant strains with tandem repeats in the promoter region of *cyp51A*. (8,9) For this reason, epidemiological studies such as the incidence of azole-resistant strains in the environment are important. Many

environmental and clinical isolates need to be screened to generate epidemiological data such as the frequency of detection of azole-resistant *A. fumigatus*. The method developed in this study would be an easy-to-use screening procedure.

Since the LAMP assay developed in the present study is one-step and rapid detection method, coupled with its high reliability and ease of use, it can be used for prompt specific detection of drug resistant genes due to TR₄₆ in *A. fumigatus* in the clinical laboratory. Early detection of infections due to TR₄₆ drug resistant strains in *A. fumigatus*, might be helpful to guide the early start of corrective and effective antifungal therapy.

MATERIALS AND METHODS

***Aspergillus* isolates.** Forty-one strains, including thirty-three from the clinical setting and eight environmental (plant bulbs) isolates (18) of *A. fumigatus* were provided through the National Bio-Resource Project (NBRP), Japan (<http://www.nbrp.jp/>); source and drug susceptibility are shown in Table 2.

DNA preparation and extraction. These fungal strains were cultured on Sabouraud dextrose agar. Genomic DNA was extracted from over-night cultures of *A. fumigatus* mycelia by urea-phenol method. Mycelia were mixed with 0.5 mm size glass beads, 0.5 ml of PCI (phenol/chloroform/Isoamyl alcohol) solution and 0.5 ml DNA extraction buffer (50 mM Tris-HCl, pH 8.0, 20 mM EDTA, 0.3 M NaCl, 0.5% SDS, 5 M urea), and disrupted by FastPrep FP100A (MP-Biomedicals, Santa Ana, USA). After centrifugation, the upper layer was transferred to a new tube and subjected to ethanol precipitation. The resulting DNA pellet was suspended in 100 µl TE buffer. DNA concentration was determined by the methods described in our previous paper (19).

MIC determination by broth microdilution test. All *A. fumigatus* strains were submitted to antifungal susceptibility tests according to the CLSI M38 protocol

(<https://clsi.org/standards/products/microbiology/documents/m38/>), using Eiken Dried Plates (9DEF47, Eiken Chemical Co., Tokyo, Japan).

LAMP-method. LAMP was performed as described in our previous studies (20). TR₄₆ LAMP primer was designed base on the target promoter region sequences of *cyp51A* gene of *A. fumigatus*, which includes tandem repeats in the promoter region containing TR₄₆ mutant alleles. The sequence information of *cyp51A* gene were downloaded from NCBI Gen-Bank (<https://www.ncbi.nlm.nih.gov/genbank>). In total, a 184-bp nucleotide alignment (Fig. 1) was used for TR₄₆ LAMP primer design by the protocol reported by Eiken Company (PrimerExplorer V5, Eiken Chemical Co. Ltd, Tokyo. Japan). LAMP primers are composed of six primers recognizing six distinct regions. The forward and backward inner primers, FIP/BIP, play crucial roles in the specificity of the assay. The outer primers, F3/B3, are composed of the fewer bases and are of a lower concentration than are FIP/BIP, initiating annealing of F3/B3 to the target in order to commence strand displacement. In addition to these four essential primers (FIP/BIP and F3/B3), the forward and backward loop primers (LF/LB) are used. The high specificity and rapidity of the present LAMP assay were achieved by applying 6 primers that target 6 regions of a DNA template, and 2 loop primers (LF, LB) to accelerate the reaction. LAMP reactions were performed with a Loopamp DNA amplification kit using reaction mixtures composed of 40 pmol each of primers FIP and BIP, 5 pmol each of primers F3 and B3, 20 pmol each of primers LF and LB, 12.5 ml ×2 reaction mixture, 1 µl Bst DNA polymerase, 2 µl DNA sample and distilled water up to a final volume of 25 µl (Eiken Chemical Co., Ltd., Tokyo, Japan) . The LAMP reactions were analyzed by a real-time turbidimeter (Loopamp EXIA; Eiken Chemical Co.) and were conducted at 63°C, for 120 min. The reaction mixtures were incubated at 61°C for 30 min (Realoop-30; Eiken Chemicals, Japan), and then heated at 80°C for 2 min to terminate the reaction. Start of amplification of LAMP products at 30 to 50 minutes in the graph, suggested the positive reaction due to the presence of corresponding a 46 bp tandem repeats of *cyp51A* gene is short. Since overall reaction can be obtained within 2 hours, prompt drug therapy can be deployed

within a short time.

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FIGURE LEGEND:

Fig. 1. Genetic information for the design of the LAMP primer set sequence.

A: Schematic illustration of *cyp51A* gene showing LAMP primer positions and corresponding sequences of TR₄₆ bp promoter tandem repeat sequences in comparison with those of wild type.

B: Primers F3, F2, F1, B1, B2 and B3 show primer sequence positions. Sequences of some primers are complementary as shown in Table 1. See LAMP primer and methods which are shown in references 19 and 20.

Fig. 2. Illustration of tandem repeat regions of *cyp51A* genes used in this experiment.

A: Tandem repeat unit of promoter genes of TR₃₄ and TR₄₆.

B: Tandem repeat: 34 bp (double) and 46 bp (double and triple), and *cyp51A* gene associated point mutation place.

Fig. 3. Comparative amplification profiles of *A. fumigatus* wild type isolates, and environmental or clinical azole resistant isolates with or without TR₄₆ double or triple 46 bp promoter repeats in *cyp51A* gene by a newly developed LAMP primer set. Amplification curve of positive control strain (IFM63432) is shown by dotted line.

A-i: DNA amplification profiles using 30 strains of *A. fumigatus* wild type. DNA amplification was not confirmed in the wild type strains tested (30 strains).

A-ii: DNA amplification was confirmed by four TR₄₆² strains (IFM 63432, BE1-2, BE1-4, BE3-5, BE3-6) which have double 46 bp promoter repeats.

A-iii: DNA amplification was confirmed by three TR₄₆³ strains (BE1-1, W1-4, W2-12-1: Y121F/M1721I/T289A/G448S) which have triple 46 bp promoter repeats. IFM63432 strain was used as a positive control.

B-I: DNA amplification was not confirmed by three TR_{46}^3 strains (BE1-1, W1-4, W212-1) which have duplicate 34 bp promoter repeats and one mutations in the coding region(L98H).

B-ii: DNA amplification was not confirmed by one TR_{34}^2 strain (3-1-B) which has duplicate 34 bp promoter repeats and multi-mutations in the coding region (L98H/T289A/I364V/G448S).

Fig. 1.

A:

Wild type -461 CATATGTTGCTCAGCGGCAGCATTCTGAAACACGTGCGTAGCAAGGGAGAAGGAAAGCACTCTGAATAATTTACACTGTT -379
TR46 CATATGTTGCTCAGCGGCAGCATTCTGAAACACGTGCGTAGCAAGGGAGAAGGAAAGCACTCTGAATAATTTACACTGTT
Primer site CATATGTTGCTCAGCGGCAG-----CACGTGCGTAGCAAGGGAGAAGGAAAGCACTCTGAATAAT---CACTGTT

F3 F2 LF

Wild type -378 CTCCTCTAGAAAAAATCATGAGTGAATAATCGCAGCACCACTTCAGAGTTGTCTAGAATCACGCGGTCCGGATGTGTGCTGA -296
TR46 CTCCTCTAGAAAAAATCATGAGTGAATAATCGCAGCACCACTTCAGAGTTGTctagaatcacggtccggatgtgtgctga
Primer site CTCCTCTAGAAAAATC-----ACCACTTCAGAGTTGTCTAGAAT----GGTCCGGATGTGTGCT--

F1 B1 LB

Wild type -295 GCCGAATGAAAGTTG-----CCTAATTACTAAG -268
TR46 gccgaatgaaagtgtTCTAGAATCACGCGGTCCGGATGTGTGCTGAGCCGAATGAAAGTTGCTAATTACTAAG
Primer site -----GAAAGTTGTCTAGAATCACGCGG-----TGTGTGCTGAGCCGAATGAAAGTTG-----

B2 B3

Fig. 2

Fig. 3

A

B

Table 1. Sequence information of newly designed and useful TR₄₆-LAMP primers set in this experiment

LAMP Primer numbers	Sequence (5' to 3')
F3	CATATGTTGCTCAGCGGCAG
B3	CAACTTTCATTCGGCTCAGCA (complementary sequence)
FIP (F1 complementary+ F2)	GAGTTTTTCTAGAGGAGAACAGTG–CACGTGCGTAGCAAGGGA (F1: complementary sequence)
BIP (B1+ B2 complementary)	CACCACTTCAGAGTTGTCTAGAAT–ACCGCGTGATTCTAGACAACTTTC (B1: Complementary)
LF	ATTATTCAGAGTGCTTCTTTCCTTC (complementary sequence)
LB	GGTCCGGATGTGTGCTG

Table 2. *Aspergillus fumigatus* strains used in this experiment

Strain No.	Isolation source	<i>cyp51A</i> genotypes	MIC values (μ g/ml)	
			ITCZ	VRCZ
IFM63432	clinical	TR ₄₆ /Y121F/T289A	4	>8
BE1-2	environmental (bulb) ^a	TR ₄₆ /Y121F/T289A	2	>8
BE1-4	environmental (bulb)	TR ₄₆ /Y121F/S363P/I364V/G448S	2	>8
BE 3-5	environmental (bulb)	TR ₄₆ /Y121F/T289A	2	>8
BE 3-6	environmental (bulb)	TR ₄₆ /Y121F/T289A	2	>8
BE 1-1	environment (bulb)	TR ₄₆ ³ /Y121F/M172I/T289A/G448S	2	>8
W1-4	environment (bulb)	TR ₄₆ ³ /Y121F/M172I/T289A/G448S	2	>8
W2-12-1	environment (bulb)	TR ₄₆ ³ /Y121F/M172I/T289A/G448S	2	>8
IFM64460	clinical	TR ₃₄ /L98H	>8	>8
IFM64733	environmental	TR ₃₄ /L98H	>8	>8
3-1-B	environment (bulb)	TR ₃₄ /L98H/T289A/I364V/G448S	2	>8
IFM62918	clinical	wild	0.5	1
IFM62799	clinical	wild	0.5	1
IFM60516	clinical	wild	1	1
IFM58402	clinical	wild	0.5	0.5
IFM51977	clinical	wild	0.25	0.25
IFM60065	clinical	wild	1	0.5
IFM61960	clinical	wild	0.5	0.5
IFM51748	clinical	wild	0.125	0.125
IFM63666	clinical	wild	1	2
IFM62520	clinical	wild	1	0.5
IFM50999	clinical	wild	0.5	0.5
IFM50268	clinical	wild	0.25	0.125
IFM55548	clinical	wild	0.25	0.25
IFM63311	clinical	wild	1	0.5
IFM63355	clinical	wild	2	2
IFM60901	clinical	wild	0.5	0.5
IFM62674	clinical	wild	1	2
IFM62709	clinical	wild	0.5	0.5
IFM52659	clinical	wild	1	1
IFM57130	clinical	wild	0.25	0.125

IFM60814	clinical	wild	0.5	0.5
IFM49435	clinical	wild	0.25	0.25
IFM61572	clinical	wild	0.5	0.5
IFM50669	clinical	wild	0.5	0.25
IFM59832	clinical	wild	0.5	0.5
IFM55044	clinical	wild	0.25	0.25
IFM47670	clinical	wild	0.5	0.5
IFM51978	clinical	wild	0.5	0.25
IFM58328	clinical	wild	0.5	0.5
IFM60369	clinical	wild	0.5	0.5

a: obtained from plant bulbs.