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

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Research Article

Keywords: rapid identification, new LAMP assay method, azole resistance, cyp51A gene, Aspergillus fumigatus

Posted Date: May 13th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-516075/v1

Development and validation of LAMP primer sets for rapid identification of *Aspergillus fumigatus* carrying the *cyp51A* TR_{46} 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 within the promoter region of the *cyp51A* gene. Loop-mediated isothermal amplification (LAMP) is a widely used nucleic acid amplification system which is fast and specific. Here

we describe a LAMP assay method to detect the 46 bp tandem repeat insertion in the *cyp51A* gene promoter region based on novel LAMP primer sets. It also differentiated strains with TR_{46} tandem repeats from those with TR_{34} tandem repeats. These results showed this TR_{46}-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 TR_{46} tandem repeats.

KEYWORDS: rapid 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 the *cyp51A* open reading frame with or without tandem repeat (TR) of 34 or 46 base pair (bp) in the promoter region of the gene (TR_{34} or TR_{46}) and overexpression of oligonucelotide sequence in the *cyp51A* gene[5]. These mutations and overexpression of the gene confer different levels of resistance[6]. Point mutations are the consequence of previous exposure to azole drugs in a clinical setting, such as for prophylaxis or therapeutic purposes[7,8]. On the other hand, TR with point mutation(s) are 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, agribusiness represents about 25% of the Brazilian Gross National Product (GNP) (http://www.agricultura.gov.br), and in this scenario, fungicide use has steadily increasing over the years. The consumption of pesticides in Brazil increased 190% in 2010 and fungicides corresponded to 14% of this (https://www.ipessp.edu.br).

In the Netherlands, prevalence of TR type resistant strains was high. Of 952 clinical *A. fumigatus* strains were collected and found to include 225 and 98 had TR_{34} and TR_{46}, respectively[10]. Another study revealed that 13 TR_{34} strains and 3 TR_{46} strains were among 105 clinical strains[10]. Additionally, there are number of reports of fatal invasive aspergillosis caused by *A. fumigatus* carrying the TR_{46} in an acute myeloid leukemia (AML) patients and hematopoietic stem cell transplant recipients[11]. Higher mortality of patients with invasive aspergillosis caused by azole resistant strain has been reported[11,12]. Thus, a rapid and specific method to identify the presence of TR would contribute to faster therapeutic decision making [13].

As one of the promising diagnostic tools for the azole resistant *A. fumigatus*, 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, lower-cost and simpler. 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 TR46（TR_{46}^2） or triple TR_{46}（TR_{46}^3）in the *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 be susceptible to itraconazole and voriconazole. The remaining 11 strains (TR_{34} and TR_{46}) were resistant to voriconazole, and most of them showed MIC values of >8μg/ml against voriconazole. Among the 11 strains, 2 strains (IFM64460 with TR34/L978H, and IFM64733 with TR34/LH98H) were resistant to itraconazole, and remaining 9 strains were susceptible to itraconazole (Table 1).

Table 1. *Aspergillus fumigatus* strains used in this experiment and their drug susceptibility profiles against itraconazole and voriconazole.

Strain No.	Isolation sources	*cyp51A* genotypes	MIC values (μg/ml)	
			ITCZ	VRCZ
IFM63432	clinic	TR_{46}^2/Y121F/T289A	4	>8
BE1-2	environment (bulb)[a]	TR_{46}^2/Y121F/T289A	2	>8
BE1-4	environment (bulb)	TR_{46}^2/Y121F/S363P/I364V/G448S	2	>8
BE 3-5	environment (bulb)	TR_{46}^2/Y121F/T289A	2	>8
BE 3-6	environment (bulb)	TR_{46}^2/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	clinic	TR_{34}/L98H	>8	>8
IFM64733	environment	TR_{34}/L98H	>8	>8
3-1-B	environment (bulb)	TR_{34}/L98H/T289A/I364V/G448S	2	>8
IFM62918	clinic	wild	0.5	1
IFM62799	clinic	wild	0.5	1
IFM60516	clinic	wild	1	1

IFM58402	clinic	wild	0.5	0.5
IFM51977	clinic	wild	0.25	0.25
IFM60065	clinic	wild	1	0.5
IFM61960	clinic	wild	0.5	0.5
IFM51748	clinic	wild	0.125	0.125
IFM63666	clinic	wild	1	2
IFM62520	clinic	wild	1	0.5
IFM50999	clinic	wild	0.5	0.5
IFM50268	clinic	wild	0.25	0.125
IFM55548	clinic	wild	0.25	0.25
IFM63311	clinic	wild	1	0.5
IFM63355	clinic	wild	2	2
IFM60901	clinic	wild	0.5	0.5
IFM62674	clinic	wild	1	2
IFM62709	clinic	wild	0.5	0.5
IFM52659	clinic	wild	1	1
IFM57130	clinic	wild	0.25	0.125
IFM60814	clinic	wild	0.5	0.5
IFM49435	clinic	wild	0.25	0.25
IFM61572	clinic	wild	0.5	0.5
IFM50669	clinic	wild	0.5	0.25
IFM59832	clinic	wild	0.5	0.5
IFM55044	clinic	wild	0.25	0.25
IFM47670	clinic	wild	0.5	0.5
IFM51978	clinic	wild	0.5	0.25
IFM58328	clinic	wild	0.5	0.5
IFM60369	clinic	wild	0.5	0.5

a: obtained from plant bulbs.

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 the *cyp51A* gene to select a set of primer sequences that specifically amplify the repeated 46 bp sequence in strains with a TR_{46} mutation (Fig. 1 and 2). To enable for specific amplification against repeated TR_{46} sequences, B2 was set on the joint of two 46 bp sequences. To obtain a specific and rapid LAMP primer set in the LAMP assay another 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 TR_{46} gene of *cyp51A* were selected, and in addition 2 loop primers (LF, LB) were also chosen to accelerate the reaction (Fig. 1). Based on the above several information, several new candidates of LAMP primers were designed and their utility tested. From those, one useful LAMP primer sets based on the detection of TR_{46} regions in *cyp51A* gene was selected (Table 2).

Fig. 1

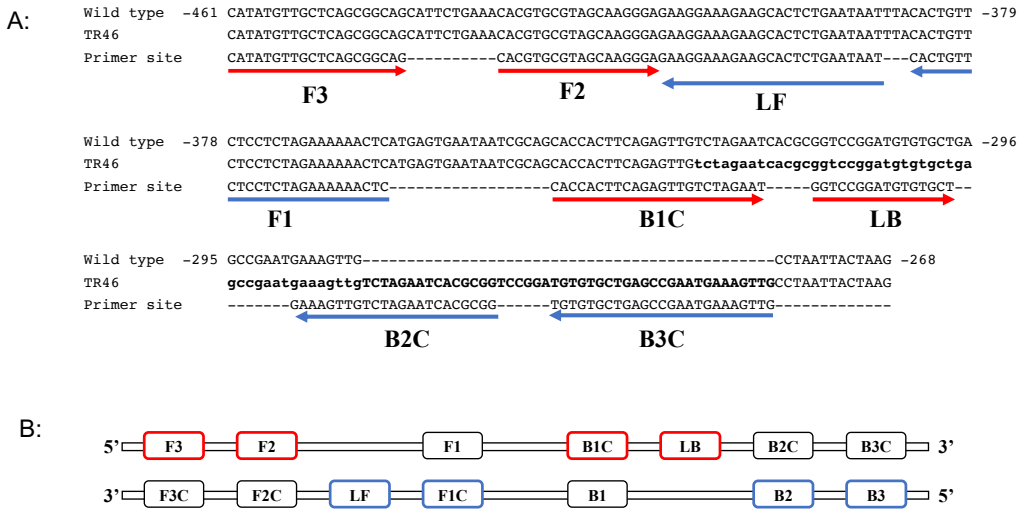

Fig. 1. Genetic information for the design of the LAMP primer sets.

A: Schematic illustration of *cyp51A* gene showing LAMP primer positions and corresponding sequences of TR46 bp promoter tandem repeat in comparion 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 2. See LAMP primer and methods which are shown in the refereces 19 and 20.

Fig. 2

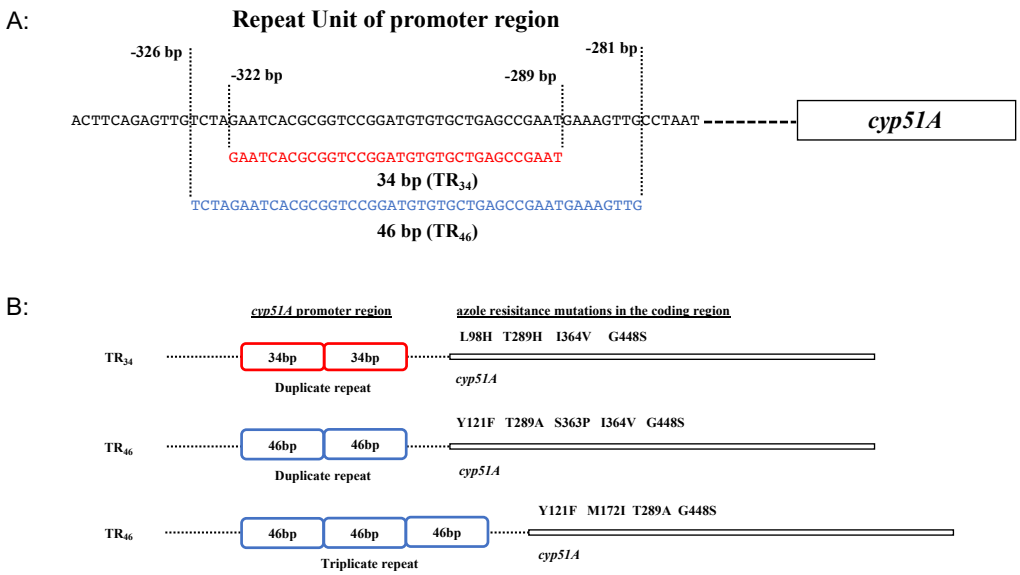

Fig. 2. Illustration of tandem repeat regions of *cyp51A* genes used in this experiment.

A: Tandem repeat unit of promoter genes of TR_{34} and TR_{46}.

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

Table 2. Sequence information of newly designed TR46-LAMP primer sets in the present experiment.

LAMP primer names	Sequence (5' to 3')
F3	CATATGTTGCTCAGCGGCAG
B3	CAACTTTCATTCGGCTCAGCA
	(complementary sequence)
FIP (F1 complementary+ F2)	GAGTTTTTTCTAGAGGAGAACAGTG-
	CACGTGCGTAGCAAGGGA
	(F1: complementary sequence)
BIP (B1 + B2 complementary)	CACCACTTCAGAGTTGTCTAGAAT-
	ACCGCGTGATTCTAGACAACTTTC
	(B1: Complementary)
LF	ATTATTCAGAGTGCTTCTTTCCTTC
	(complementary sequence)
LB	GGTCCGGATGTGTGCTG

Validation of LAMP primer sets for TR_{46}

Specificity of the primer sets was tested using various types of *A. fumigatus* strains, such as wild isolates, and environmental or clinical azole resistance isolates (Fig. 3). In this study, IFM 63432 strain was used as a positive control. As shown in Fig. 3A-i, TR46 LAMP primer could not amplify the DNA from 30 strains of azole drug susceptible clinical isolates of *A. fumigatus*. However, the start of the LAMP amplification in the control strain of *A. fumigatus* strains (IFM 63432) was at around 50 min. On the other hands, TR_{46} LAMP primer could amplify DNA from *A. fumigatus* strains carrying the duplicate 46 bp promoter repeat in *cyp51A* gene

(IFM63432, Be1-2, BE1-4, BE3-5, BE3-6) as shown in Fig. 3A-ii. This result suggests that the present LAMP primer could amplify the four TR_{46}^2 strains harboring the TR46 resistant mutation (TR46/Y121F/T289A). It was also confirmed that TR_{46} LAMP primer could amplify DNA of *A. fumigatus* strains carrying three TR_{46}^3 strains (BE1-!, W1-4, W2-12-1) (Fig.3A-iii). When this TR_{46} LAMP primer was tesed for three TR_{34}^2 strains (Table 1), namely strains IFM64460 and 64733 (with mutation of TR34/L98H) and strain 3-1-B (with mutations of TR_{34}/L98H/Y289/T289A/I364V/G448S), DNA amplification was not observed (Fig. 3B-i and B-ii). These results also suggested that the present LAMP primer could not detect TR_{34}^2 drug resistant strains regardless of their point mutation site in *cyp51A* gene (Fig. 3 B-i and B-ii). These studies confirmed that newly established TR_{46} LAMP primer set was specific *A. fumigatus* strains with TR of double or triple 46-bp promoter tandem repeats in *cyp51A* gene.

Fig. 3

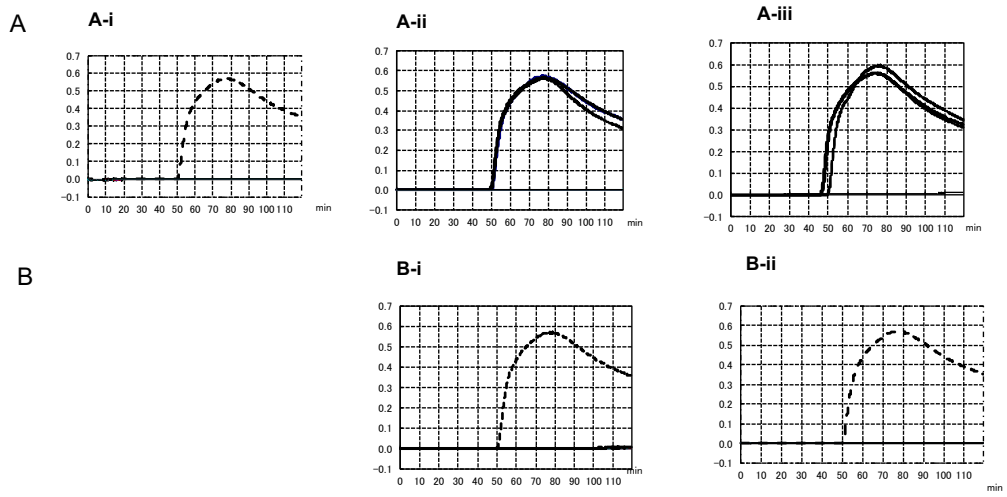

Fig. 3. Comparative amplification profiles of *A. fumigatus* wild type, and environmental or clinical azole resistant isolates with or without TR46 double or triple 46 bp promoter repeats in *cyp51A* gene by a newly developed LAMP primer sets. Dodded curve shows

the amplification by control strain (IFM 63432).

A-i: DNA amplification profiles using 30 strains of *A. fumigatus* wild type. DNA amplification was not comfirmed in all wild type strains tested (30 strains). Dodded curve shows amplification by control strain.

A-ii: DNA amplification was comfirmed by five TR_{46}^{2} strains (IFM63432, BE1-2, BE1-4, BE3-5, BE3-6) which have double 46 bp promoter repeats.

A-iii: DNA amplification was confirmed by three TR_{46}^{3} strains (BE1-1, W1-4, W2-12-1) which have triple 46 bp promoter repeats.

B-I: DNA amplification was not confirmed by two TR_{34}^{2} strains (IFM64460, IFM64733) which have duplicate 34 bp promoter repeats with one mutations in the one coding region (L98H). Dodded line shows amplification by control strain.

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

Discussion

Azole antifungals mainly inhibit the 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. 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 TR_{46} gene.

Recently Yu Shan-Ling *et al.*[15] reported a similar rapid technique to detect azole resistant strains due to amplification of a TR of a 34 bp (TR_{34}) and a 46 bp (TR_{46}) within the promoter region of *cyp51A* of *A. fumigatus.* However, here we used a newly designed TR46 LAMP primer sets which are different from those reported by Yu Shan-Ling *et al*.[15].

There is a difference in MIC values between strains with TR_{46} and strains with TR_{34}[16]. Thus, the importance of detecting TR_{46} lies in the fact that strains of *A. fumigatus* harboring TR_{46} are resistant to voriconazole, but not to itraconazole, while two TR_{34} strains (IFM64460: TR34/L98H and IFM64733: TR34/L98H) are highly resistant to voriconazole[16] but not to itraconazole. Further detail drug susceptibility mechanism study against TR_{34} strain (3-1-B: TR34L98H/T289A/I364V/G448S) is of interest.

In this study we succeeded in the design of useful TR_{46} LAMP primer sets to detect specifically a TR_{46} within the promoter regions of azole resistant *A. fumigatus*. The designed primer sets could differentiate azole resistant TR_{46} strains from that of TR_{34} strains. To our knowledge, this is a new and useful report of a detection method for one of the most prevalent *cyp51A* resistant gene TR_{46} in *A. fumigatus* azole resistant strains.

Recently, the strain consisting of the four repeats of 46 bp of the promoter region was reported in the Netherlands (TR_{46}^4)[17]. The LAMP primer we designed was able to detect both two copies of the TR46 tandem repeat and three copies of the TR_{46}. 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 such as TR_{46}[4].

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* gene[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 TR_{46} in *A. fumigatus* in the clinical laboratory. Early detection of infections due to TR_{46} drug resistant strains in *A. fumigatus,* might be helpful to guide the early start of corrective and effective antifungal therapy.

Methods

Aspergillus isolates and MIC determination by broth microdilution test

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 1.

DNA preparation and extraction

Fungal strains were cultured on Sabouraud dextrose agar. Genomic DNA was extracted from

over-night cultures of *A. fumigatus* mycelia by the 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 Fast Prep 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].

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]. TR_{46} LAMP primer was designed based on the target promoter region sequences of *cyp51A* gene of *A. fumigatus*, which includes tandem repeats in the promoter region containing TR_{46} mutant alleles. The sequence of the *cyp51A* gene were downloaded from NCBI Gen-Bank (https://www.ncbi.nlm.nih.gov/genebank). In total, a 184-bp nucleotide alignment (Fig. 1) was used for TR_{46} LAMP primer design by the protocol of the Eiken Company (Primer Explorer 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℃ 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.

Acknowledgements

This study was supported by AMED under Grant Numbers JP20jm0110015 and by JICA through the collaborative research project Science and Technology Research Partnership for Sustainable Development (SATREPS), Japan.

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Table Title

Table 1. *Aspergillus fumigatus* strains used in this experiment and their drug susceptibility profiles against itraconazole and voriconazole.

Table 2. Sequence information of newly designed TR46-LAMP primer sets in the present experiment.

Figure legend

Figure 1. Genetic information for the design of the LAMP primer sets.

A: Schematic illustration of *cyp51A* gene showing LAMP primer positions and corresponding sequences of TR46 bp promoter tandem repeat in comparion 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 2. See LAMP primer and methods which are shown in the refereces 19 and 20.

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

A: Tandem repeat unit of promoter genes of TR_{34} and TR_{46}.

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

Figure 3. Comparative amplification profiles of *A. fumigatus* wild type, 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 sets. Dodded curve shows the amplification by control strain (IFM 63432).

A-i: DNA amplification profiles using 30 strains of *A. fumigatus* wild type. DNA amplification was not comfirmed in all wild type strains tested (30 strains). Dodded curve shows amplification by control strain.

A-ii: DNA amplification was comfirmed by five TR_{46}^{2} strains (IFM63432, BE1-2, BE1-4, BE3-5, BE3-6) which have double 46 bp promoter repeats.

A-iii: DNA amplification was confirmed by three TR_{46}^{3} strains (BE1-1, W1-4, W2-12-1) which have triple 46 bp promoter repeats.

B-I: DNA amplification was not confirmed by two TR_{34}^{2} strains (IFM64460, IFM64733) which have duplicate 34 bp promoter repeats with one mutations in the one coding region (L98H). Dodded line shows amplification by control strain.

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

Figures

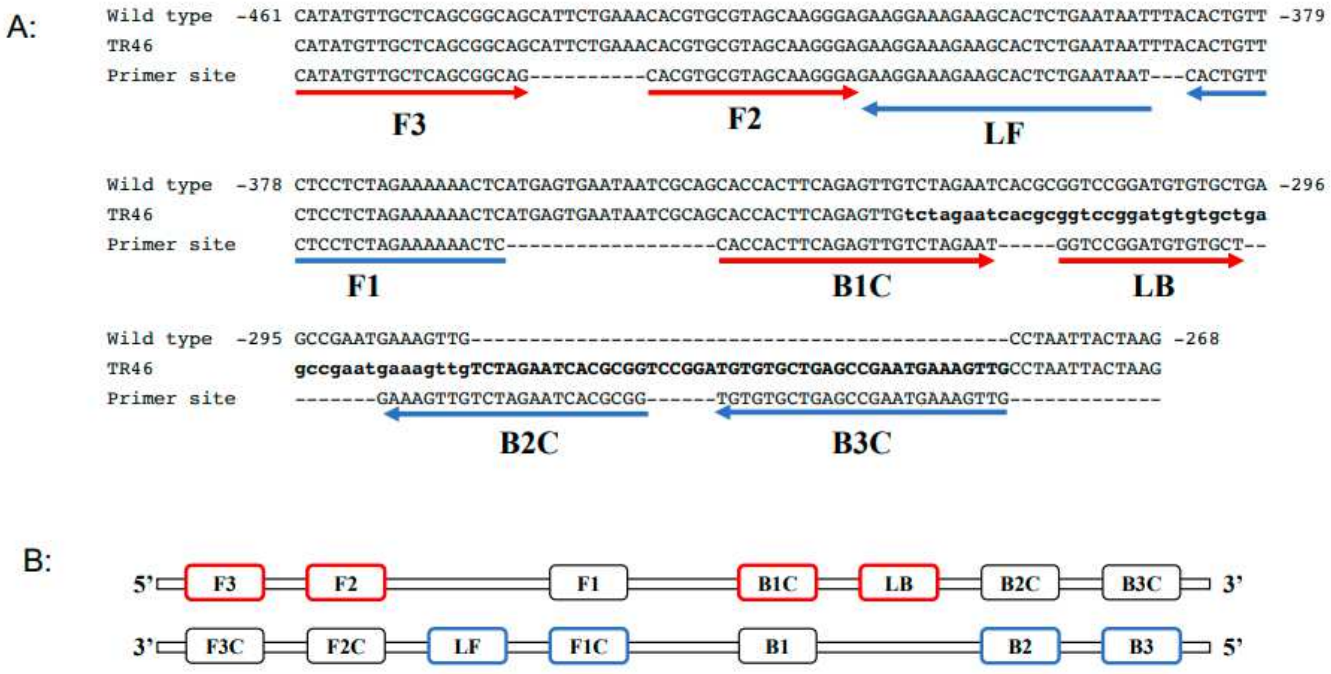

Figure 1

Please see the Manuscript PDF file for the complete figure caption

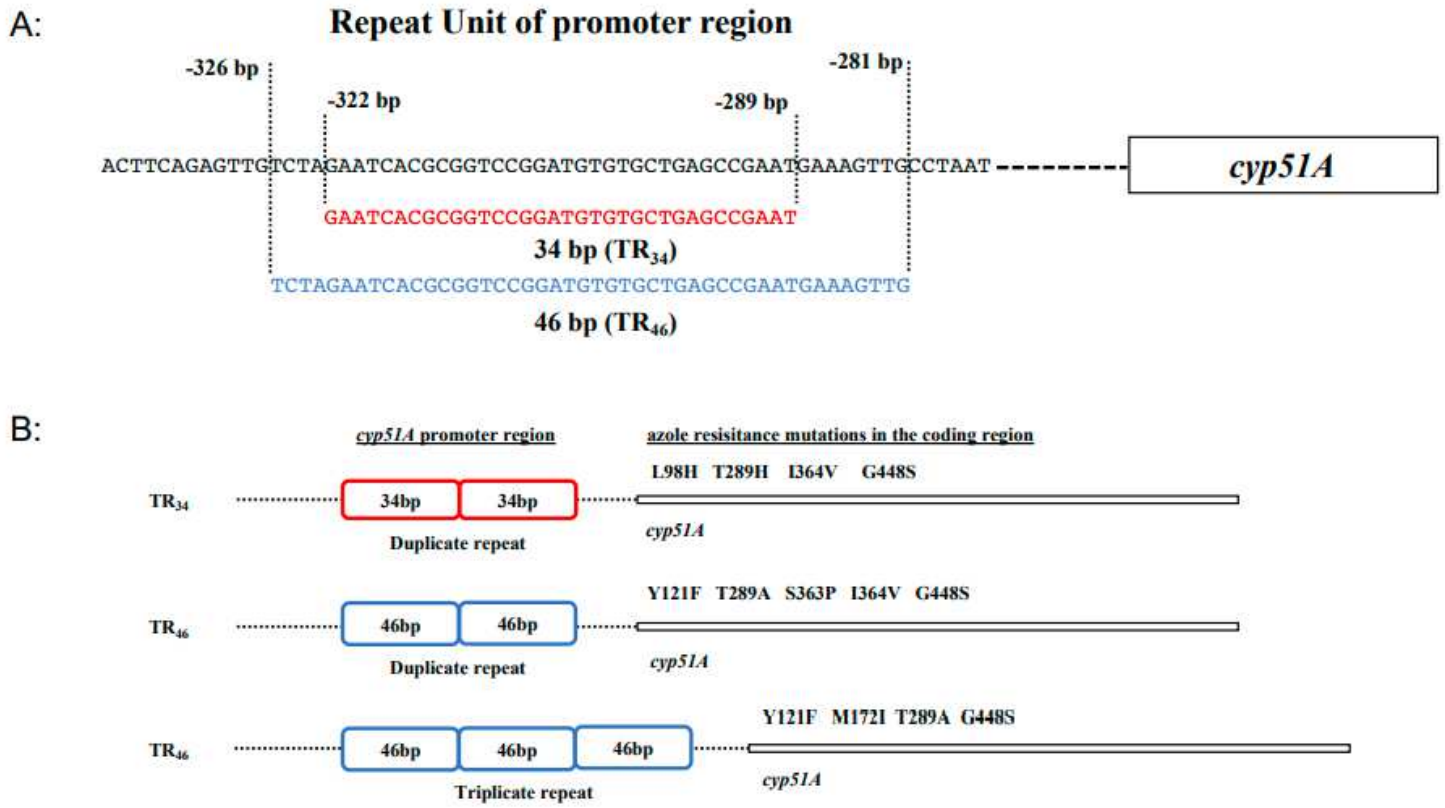

Figure 2

Please see the Manuscript PDF file for the complete figure caption

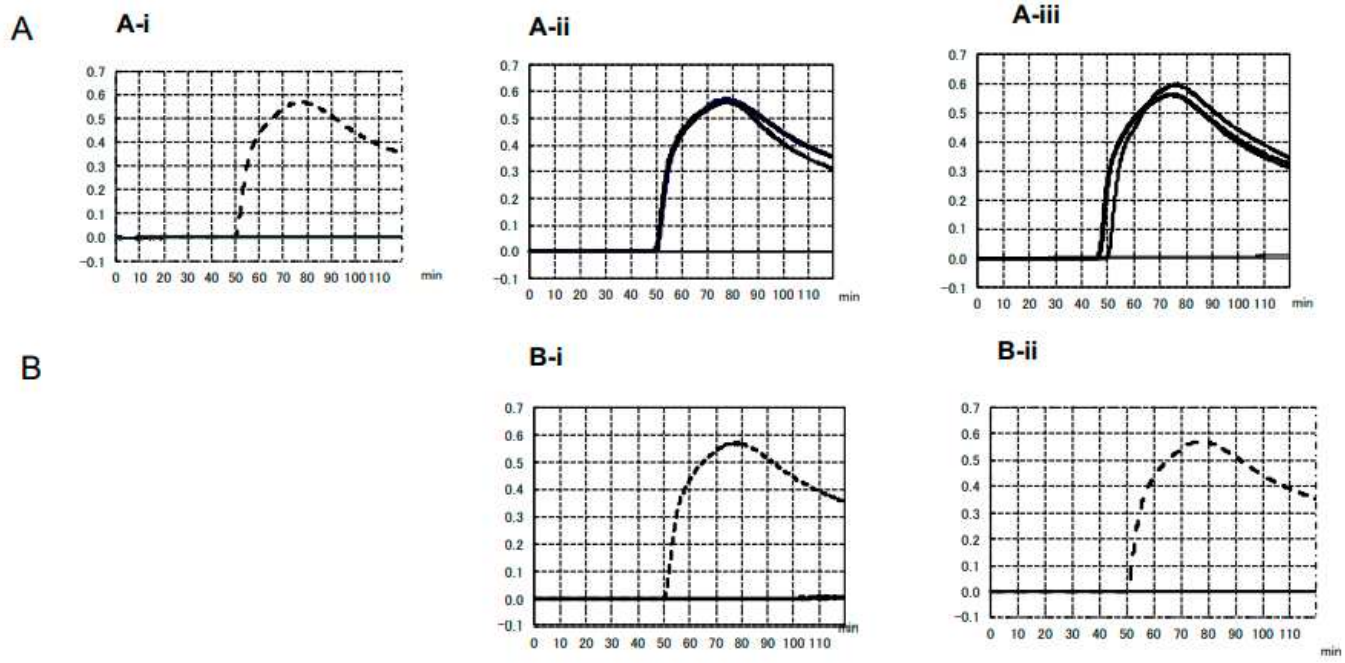

Figure 3

Please see the Manuscript PDF file for the complete figure caption