A simple method to detect the tandem repeat of the *cyp51A* promoter in azole-resistant strains of *Aspergillus fumigatus*

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ABSTRACT

We designed primers and cycling probes to detect the tandem repeat (TR) of *cyp51A* promoter in *Aspergillus fumigatus*. A control-probe was designed to anneal to the outside of the TR region, whereas a TR-probe was designed to anneal to the inside of the TR region. For amplification and probe-hydrolysis detection, CycleavePCR system was used. Although the difference between Ct values of the wild-type genome for the control-probe and the TR-probe was around −0.1, the difference between Ct values of TR-harboring strains was around 0.7. These data indicate that this is a simple method to detect TR in azole-resistant *A. fumigatus*.

Azole-resistant strains of *Aspergillus fumigatus* in the environment are a growing problem across the world, including in Japan[1–3]. These strains contain a 34-bp, 46-bp, or 53-bp tandem repeat (TR) in the *cyp51A* promoter in *A. fumigatus*. Recently, three or four 46-bp repeats have been found in environmental and/or clinical isolates[4]. These repeats are a good target to differentiate azole-resistant strains from wild-type strains. In the present study, we proposed a simple and convenient method to detect TR in resistant strains of *A. fumigatus*.

For the assay, we designed four oligonucleotides (Table). Two of the four oligonucleotides, TR-F and TR-R, were the primers used to amplify the *cyp51A* promoter region (approximately 120 bp in wild-type genome), including the TR region in azole-resistant strains (Table and Figure a). The other two oligonucleotides were cycling probes, which is a variety of hydrolysis probes (Table). We designed a TR-probe to anneal to the inside of the TR region (Figure a) and a positive control (PC)-probe to anneal to the outside of the TR region (Figure a). The PC-probe was labeled with HEX fluorophore and used as the positive control. The TR-probe was labeled with FAM fluorophore and used as the indicator of TR.

We prepared PCR fragments from *A. fumigatus* OKH31 and OKH50 strains[3,5] amplified with TR-F and TR-R primers. *A. fumigatus* OKH50 is an azole-resistant strain harboring TR_{34} in the *cyp51A* promoter. The copy numbers of the prepared PCR fragments from OKH31 and OKH50 were estimated as 4×10^9 and 5×10^9 copies/μl, respectively. These fragments were diluted 10, 10^2, 10^3, 10^4, 10^6, or 10^8 times with ultrapure water and

51 then applied to real-time PCR as templates. The procedure of real-time PCR is shown in
52 Figure b. Briefly, the PCR reaction mixture included 1 μL of template DNA, 0.2 μM each
53 of TR-F and TR-R primers, 0.2 μM each of TR- and PC-probes, and 20 μL of
54 CycleavePCR Reaction Mix (Takara Bio Inc., Shiga, Japan). LightCycler 480 Instrument II
55 (Roche Diagnostics K.K., Tokyo, Japan) was used for the amplification and detection of
56 hydrolysis. The reaction was performed by denaturation at 95 °C for 30 s, followed by 45
57 cycles of denaturation at 95 °C for 5 s, annealing at 55 °C for 10 s, and polymerization and
58 detection at 72 °C for 15 s.

59 ΔCt values calculated as $Ct_{PC} - Ct_{TR}$, ranged from −0.22 to 0.04 for the fragment
60 from OKH31, indicating equivalent levels of both PC and TR regions in the template DNA.
61 In contrast, ΔCt values for OKH50 were around 0.6 or 0.7 (Figure c), indicating that the
62 template DNA contained additional copy of the TR region compared with the PC region.
63 The difference between ΔCt values of OKH31 and OKH50 was around 0.8, indicating that
64 this method could differentiate TR-containing region from that of the wild type. Under the
65 use of the smallest copy number (<100 copies) in this experiment, large variations were
66 detected and ΔCt differences were smaller than those under the higher copy numbers
67 because of unknown reason (Figure c), which suggests that pre-amplification is useful for
68 samples containing a small a copy number of genomic DNA.

69 Furthermore, we used genomic DNA from *A. fumigatus* OKH31, OKH50, and
70 IFM634321, a TR_{46}/Y121F/T289F strain, for the method. As shown in Figure d, the ΔCt
71 values of the OKH31 genome were around −0.1. In contrast, the ΔCt values of OKH50 and

IFM63432 strains were around 0.7. Genomic DNA from 12 other wild-type *A. fumigatus* strains also indicated the ΔCt values between −0.41 and −0.1 (data not shown). These data suggest that this method can be applied for TR detection from genomic DNA.

To summarize, we proposed a method to detect TR of the *cyp51A* promoter in azole-resistant strains. Multiplex real-time PCR assays are commercially available and are useful for the rapid identification of azole-resistant strains harboring TR as well as other point mutations. Our method provides another tool to identify azole-resistant strains with TR. Utilizing our method, the TR-probe can detect not only TR_{34}-harboring strains but also TR_{46}-harboring strains. Differentiation between TR34-harboring strains and TR46-harboring strains is important because resistance patterns between both strains are different. Cycling probes are useful to detect SNP; therefore, we are trying to prepare additional probes to differentiate between L98H and Y121F/T289F. We suggest that additional panels to TR detection are useful to differentiate between TR strains. The proposed method has another advantage in that the PC-probe and TR-probe anneal to and are consumed on the same amplicon; as a result, the Ct difference is not affected by the initial copy number of genome DNA and delay in amplification by inhibitory substances in the reaction mixture. Although this method needs to be validated using DNA from multiple strains and clinical specimens, this simple method was provided as another tool for TR detection in *A. fumigatus*.

Acknowledgments

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References

1. Hagiwara D, Takahashi H, Fujimoto M, et al. Multi-azole resistant *Aspergillus fumigatus* harboring Cyp51A TR_{46}/Y121F/T289A isolated in Japan. *J Infect Chemother*. 2016; 22: 577-579.
2. Onishi K, Sarumoh BM, Hagiwara D, Watanabe A, Kamei K, Toyotome T. Azole-resistant *Aspergillus fumigatus* containing a 34-bp tandem repeat in *cyp51A* promoter is isolated from the environment in Japan. *Med Mycol J*. 2017; 58: E67-E70.
3. Toyotome T, Hagiwara D, Kida H, et al. First clinical isolation report of azole-resistant *Aspergillus fumigatus* with TR_{34}/L98H-type mutation in Japan. *J Infect Chemother*. 2017; 23: 579-581.
4. Zhang J, Snelders E, Zwaan BJ, et al. A novel environmental azole resistance mutation in *Aspergillus fumigatus* and a possible role of sexual reproduction in its emergence. *mBio*. 2017; 8: 1-13.
5. Toyotome T, Fujiwara T, Kida H, Matsumoto M, Wada T, Komatsu R. Azole susceptibility in clinical and environmental isolates of *Aspergillus fumigatus* from eastern Hokkaido, Japan. *J Infect Chemother*. 2016; 22: 648-650.

Figure legend

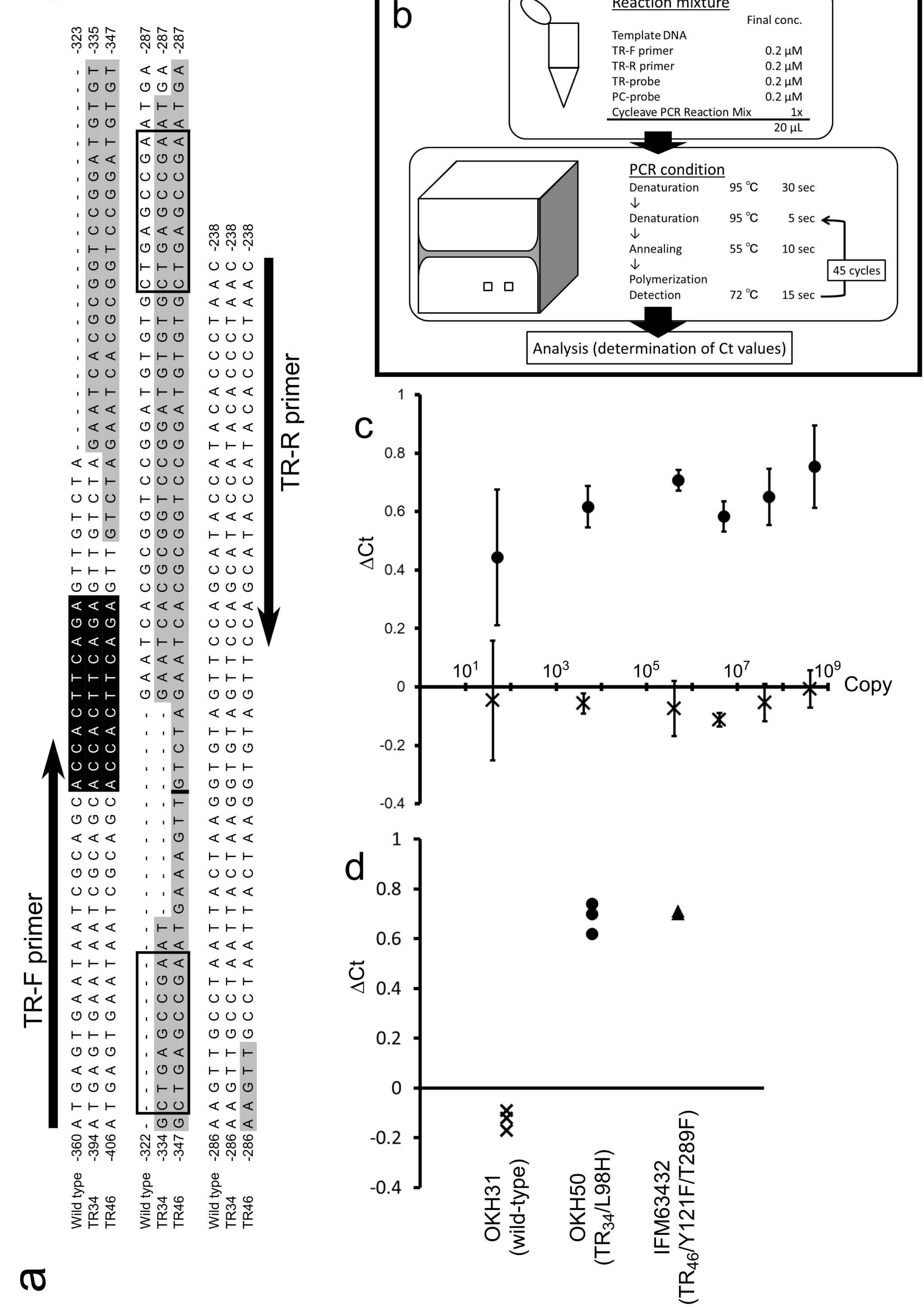
Figure. (a) The region of wild-type, TR_{34}, and TR_{46} strains amplified by TR-F and TR-R primers whose annealing sites are indicated by arrows in the figure. Characters on the black background indicate the control-probe binding site. Boxed sites are TR-probe binding sites. Gray-background nucleotides indicate the tandem repeats of TR_{34} and TR_{46} strains. (b) Condition and procedure of real-time PCR analysis. (c) Differences of Ct values between the PC-probe and TR-probe. Circles and X marks indicate each mean of differences of Ct values from the TR_{34} strain OKH50 and wild-type strain OKH31, respectively. Each experiment was repeated three times. Error bars indicate standard deviation. (d) Differences of Ct values between the PC-probe and TR-probe using genomic DNA. X marks indicate the differences of the OKH31 strain. Filled circles and triangles indicate the differences of OKH50 and IFM63432, respectively. Each experiment was repeated three times.

Table. Oligo DNA and cycling probes used in this study

Name	Sequence (5′ → 3′)
TR-F	ATGAGTGAATAATCGCAGCACC
TR-R	GTTAGGGTGTATGGTATGCTGG
TR-probe[†]	Eclipse-CTG(A)GCCGA-FAM
Control-probe[†]	Eclipse-TCTG(A) AGTGGT-HEX

[†]Parentheses indicate RNA residues. Probes were conjugated with a quencher Eclipse to the 5′-end and a fluorophore FAM or HEX to the 3′-end.

Figure



a
TR-F primer
TR-R primer
Wild type
TR34
TR46
b
Reaction mixture
Final conc.
Template DNA
TR-F primer 0.2 µM
TR-R primer 0.2 µM
TR-probe 0.2 µM
PC-probe 0.2 µM
Cycleave PCR Reaction Mix 1x
20 µL
PCR condition
Denaturation 95 °C 30 sec
Denaturation 95 °C 5 sec
Annealing 55 °C 10 sec
Polymerization
Detection 72 °C 15 sec
45 cycles
Analysis (determination of Ct values)
c
ΔCt
Copy
d
ΔCt
OKH31 (wild-type)
OKH50 (TR_{34}/L98H)
IFM63432 (TR_{46}/Y121F/T289F)