Azole susceptibility in clinical and environmental isolates of *Aspergillus fumigatus* from eastern Hokkaido, Japan

Takahito Toyotome[a, b], Takayuki Fujiwara[a], Hideyuki Kida[c], Munehiro Matsumoto[d], Terumi Wada[c], and Ryoichi Komatsu[c]

[a] Diagnostic Center for Animal Health and Food Safety, Obihiro University of Agriculture and Veterinary Medicine, Nishi 2-11, Inada-cho, Obihiro, Hokkaido, 080-8555, Japan

[b] Division of Clinical Research, Medical Mycology Research Center, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba City, Chiba 260-8673, Japan

[c] Department of Medical Technologist, Obihiro-Kosei General Hospital, West 6 South 8-1, Obihiro, Hokkaido, 080-0016, Japan

[d] First Department of Internal Medicine, Obihiro-Kosei General Hospital, West 6 South 8-1, Obihiro, Hokkaido, 080-0016, Japan

Correspondence:

Takahito Toyotome

Diagnostic Center for Animal Health and Food Safety, Obihiro University of Agriculture and

19 Veterinary Medicine, Nishi 2-11, Inada-cho, Obihiro, Hokkaido, 080-8555, Japan

20 TEL/FAX: +81-155-49-5888

21 E-mail address: tome@obihiro.ac.jp

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Abstract

Azole antifungals are used not only clinically for fungal infections but also used as agricultural fungicides. Recently, azole-resistant *Aspergillus fumigatus* containing a tandem repeat in the promoter region of *cyp51A* combined with amino acid substitution(s) appears in the environment in Eurasia, especially in several European countries. Although azole fungicides have been used in Japan, especially in Hokkaido, surveillance and characterization of *A. fumigatus* in Hokkaido have not been reported. In this study, we collected soil samples from farms that used an azole fungicide in the Tokachi area of eastern Hokkaido, isolated 91 *A. fumigatus* strains, and determined the minimal inhibitory concentrations of medical azoles required for these strains. Moreover, because causative agent *A. fumigatus* is ubiquitous in the air and acquired from the environment, we collected 22 clinical isolates of *A. fumigatus* to measure their susceptibility to medical azoles in a hospital in the Tokachi area. Our data show that almost all *A. fumigatus* isolates retained susceptibility to medical azoles. Clinical isolates OKH34 and OKH6 showed 8 and 2 μg/mL of voriconazole, respectively, as the minimal inhibitory concentration. Both isolates did not contain tandem repeat in *cyp51A* promoter region. An isolate contained G448S mutation in *cyp51A* conferring voriconazole resistance, which is the first report from Japan. Our data shows the existence of azole-resistant and low azole-susceptible clinical isolates and highlight the necessity for continuous surveillance in

Japan because resistant *A. fumigatus* strains can arise through clinical or environmental selection or could be introduced from overseas.

Keywords: azole, *Aspergillus fumigatus*, *cyp51A*

Aspergillus fumigatus is a fungus commonly found in the environment, known to be the leading causative agent of aspergillosis. Azole antifungals are used not only to treat aspergillosis and other fungal infections but are also used as agricultural fungicides. Because of the extensive use of azole antifungals, azole-resistant *A. fumigatus* strains have been reported globally (reviewed in [1]). Although some *A. fumigatus* strains are intrinsically resistant to azoles, acquired resistant strains have also been reported [1, 2]. The mechanisms responsible for the acquisition of azole resistance are roughly classified into the selections in a patient and in the environment [2]. Tashiro and colleagues have reported the correlation between the duration of azole treatment and the susceptibility of *A. fumigatus* isolates [3], indicating that long-term exposure of azoles in patients increases the likelihood of resistance acquisition. Alternatively, recent studies and evidence suggest that the use of azoles fungicides has driven the acquisition of azole resistance (reviewed in [4]). Most environmental isolates with azole resistance harbored the *cyp51A* mutation consisting of a tandem repeat in the *cyp51A* promoter region and amino acid substitution(s) named as TR_{34}/L98H or TR_{46}/Y121F/T289A [1].

As of 2015, 17 azole fungicides have been approved for agricultural use in Japan. The shipment of tebuconazole was the largest among azole fungicides in Japan from October 2011 to September 2012 (2012 agricultural chemical year [5]). Snelders and colleagues

reported that epoxiconazole, difenoconazole, propiconazole, bromuconazole, and tebuconazole exhibited similar docking poses to medical triazoles [6]. Of these five fungicides, tebuconazole, propiconazole, and difenoconazole were placed first, second, and fourth, respectively, in terms of their usage in the 2012 agricultural–chemical year in Japan. These three fungicides were mainly shipped to Hokkaido [5]. Recently, Kano and colleagues examined the azole susceptibility of *A. fumigatus* isolates from a farm in Japan that used tetraconazole twice a year for over 15 years [7]. The study showed that the isolated strains did not exhibit resistance to tetraconazole or itraconazole (ITCZ). In this study, we examined the azole susceptibility in environmental isolates of *A. fumigatus* from the Tokachi area of eastern Hokkaido. In addition to the examination of environmental isolates, we examined the azole susceptibility of clinical isolates collected at a hospital in the Tokachi area, because the causative agents of aspergillosis are acquired from the environment.

Soil samples listed in Supplementary Table 1 were collected in the Tokachi area of eastern Hokkaido, Japan. We identified *A. fumigatus* by macroscopic observation and subcultured it on PDA. To determine minimal inhibitory concentrations (MIC) of medical antifungals excluding micafungin and minimal effective concentrations (MEC) of micafungin, the broth microdilution method based on CLSI M38-A2 with a slight modification were used. In brief, conidia were diluted in 100 μL of MOPS(NACALAI TESQUE, Inc., Kyoto,

Japan)-buffered RPMI 1640 medium (Sigma-Aldrich Co., St. Louis, MO) adjusted to pH 7.0 at 1×10^4 cells/mL, and then were inoculated to each well of 96-well microplates, containing medical antifungals at various concentrations (Dry Plate Eiken; Eiken Chemical Co., Ltd., Tokyo, Japan). After 48 h of culture at 35°C, the growth inhibition of mold was determined with visual observation. MIC and MEC of antifungals against isolates from soil samples was shown in Table 1. All of 91 isolates from these soil samples were susceptible to medical azoles. These results suggest that TR34/L98H- or TR46/Y121F/T289A-type were not prevalent in Tokachi area of eastern Hokkaido.

Twenty-two of *A. fumigatus* strains were isolated from clinical specimens in Obihiro-Kosei General Hospital in the Tokachi area from November, 2013 to July, 2015 (Supplementary Table 2). To collect and use of these clinical isolates, we received an approval (2013-02) from the ethics committee of Obihiro University of Agriculture and Veterinary Medicine and an approval from the ethics committee of Obihiro-Kosei General Hospital. Conidia of these strains were stocked as glycerol stocks in −80°C until using. Genomic DNA were extracted from these clinical isolates and identified as *A. fumigatus* by the determination of Internal transcribed spacer and D1/D2 regions. Additionally, partial nucleotide sequences of β-tubulin, *rodA*, and calmodulin genes of OKH6 and OKH34 clinical isolates were determined. Primers used for amplification and sequencing are listed in

Supplementary Table 3. Twenty-two clinical isolates identified as *A. fumigatus* were determined the MIC of antifungals (Table 2). MIC of VRCZ against OKH6 and OKH34 were determined at least three times on different days. Although almost all of these isolates were susceptible to antifungals including azoles, the isolate, *A. fumigatus* OKH6, exhibited low susceptibility to voriconazole (VRCZ) (MIC 2 μg/mL). Because MIC of ITCZ against *A. fumigatus* OKH6 was 1 μg/mL, the strain did not exhibit cross-resistance to ITCZ. And among the remaining 21 strains of *A. fumigatus*, OKH34 exhibited resistance to VRCZ (MIC 8 μg/mL). The strain OKH34 also did not exhibit cross-resistance to ITCZ (MIC 0.5 μg/mL). These results indicate that among 22 clinical isolates collected in this study two isolates OKH6 and OKH34 decreased the susceptibility to VRCZ.

To compare *cyp51A* and the upstream regions of *A. fumigatus* OKH6, OKH34 and other azole susceptible *A. fumigatus* strains (Af293, OKH1 and OKH2), we determined these nucleotide sequences. To amplify and determine the nucleotide sequences of *cyp51A* with the upstream region, primers listed in Supplementary Table 3 were used. As shown in Supplementary Figure 1a, the coding sequence of *cyp51A* in OKH6 strain was identical to those in OKH1 and OKH2 strains. On the other hand, OKH34 strain has a unique amino acid substitution from glycine to serine in the residue 448 of *cyp51A*. In the coding sequence of *cyp51B*, OKH6, but not OKH1 and OKH2, has P327S mutation (Supplementary Figure 1b).

The 1kb upstream region of *cyp51A* of OKH6 did not contain tandem repeats. We examined the expression level of *cyp51A* and *cyp51B* in OKH6 and compared with the expression levels in OKH1 and OKH2. After statically culturing for 48 h at 35°C in MOPS-RPMI medium, total RNA were recovered from mycelia with Direct-zol RNA MiniPrep kit with TRI-Reagent (Zymo Research Irvine, CA). Real-time quantitative PCR was performed using THUNDERBIRD SYBR qPCR Mix (Toyobo Co., Ltd., Osaka, Japan) and LightCycle 480 II (Roche Diagnostics, Basel, Switzerland). Primers were listed in Supplementary Table 3. The expression levels of *cyp51A* and *cyp51B* in OKH6 were comparable to those of OKH1 and OKH2 (Figure 1). These results indicate that the G448S mutation in *cyp51A* contributed to resistance of OKH34 to VRCZ.

cyp51A of *A. fumigatus* OKH34 possessed a mutation at glycine residue 448 to serine. The point mutation has been described several manuscripts [8-12], but the mutant has not been reported from Japan. Clinical isolates including G448S mutation described in several manuscripts were resistant to VRCZ and ITCZ [9-11]. In Japan, to the best of our knowledge, the mutant has not been reported. On the other hand, G448S mutants obtained by in vitro-selection were resistance to voriconazole [8]. Our data also indicated that G448S mutant conferred resistance to voriconazole. The patient had received treatment with VRCZ (200 mg daily), and on day 85 after starting VRCZ treatment *A. fumigatus* OKH34 was

isolated from a sputum specimen. Before the antifungal treatment, an *A. fumigatus* strain OKH31 was isolated from the patient and was susceptible to VRCZ, as well as ITCZ. This mutation, therefore, might have been induced during this treatment. Another strain, OKH6, showed low sensitivity to VRCZ and the *cyp51A* sequence did not contain any mutation, contributing to azole resistance, and the expression levels of *cyp51A* and *cyp51B* did not changed in OKH6. In *cyp51B* coding sequence of OKH6, we found P327S mutation. Since the mutation has not been reported, the importance remains unknown. Recently, the structure of *A. fumigatus* Cyp51B was resolved [13], indicating that P327-residue located far from the active site of Cyp51B. Therefore, the mutation might not be related the VRCZ low susceptibility. Further investigation is required to elucidate a role of the P327S mutation to azole resistance.

In conclusion, we isolated *A. fumigatus* from soil samples obtained from the Tokachi area, and all of the isolates were susceptible to medical azoles. These results suggest that TR34/L98H- or TR46/Y121F/T289A-type were not prevalent in Tokachi area of eastern Hokkaido. Among clinical isolates, we found two *A. fumigatus* strains with low VRCZ susceptibility. However, these isolates did not possess tandem repeats in the upstream region of *cyp51A*. Although Wiederhold et al. reported that TR34/L98H- or TR46/Y121F/T289A-type strains were isolated sporadically in U.S. since 2008, these type

resistant strains have not been recovered from the environment in U.S. [14] Very recently, a TR46/Y121F/T289A-type resistant strain was clinically isolated from a patient in Japan [15]. In this situation of sporadic isolation, these resistant strains might not be prevalent and might not be isolated from the environment. *A. fumigatus* TR34/L98H- or TR46/Y121F/T289A-type resistant strain may appear in the environment in Japan through inadequate use of azole pesticides or by importing agricultural products from overseas. For these reasons, we need continuous surveillance of *A. fumigatus* isolates in Japan, including Hokkaido.

Acknowledgments

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Conflict of Interest

None

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Figure 1: The relative quantification of *cyp51A* (black bars) and *cyp51B* (white bars) in *A. fumigatus* OKH1, OKH2, and OKH6 isolates.

Supplementary Figure 1: Nucleotide sequences of *cyp51A* (a) and *cyp51B* (b) of *A. fumigatus* Af293 strain and OKH6, OKH1, OKH2, and OKH34 isolates. Grey-shaded sites indicate that the nucleotide of at least one OKH strain is different from the Af293 strain. Boxed sites in (a) and (b) indicate the codon at residue 448 of *cyp51A* and at residue 327 of *cyp51B*, respectively. Coding sequences are underlined in the figure.

Figure 1

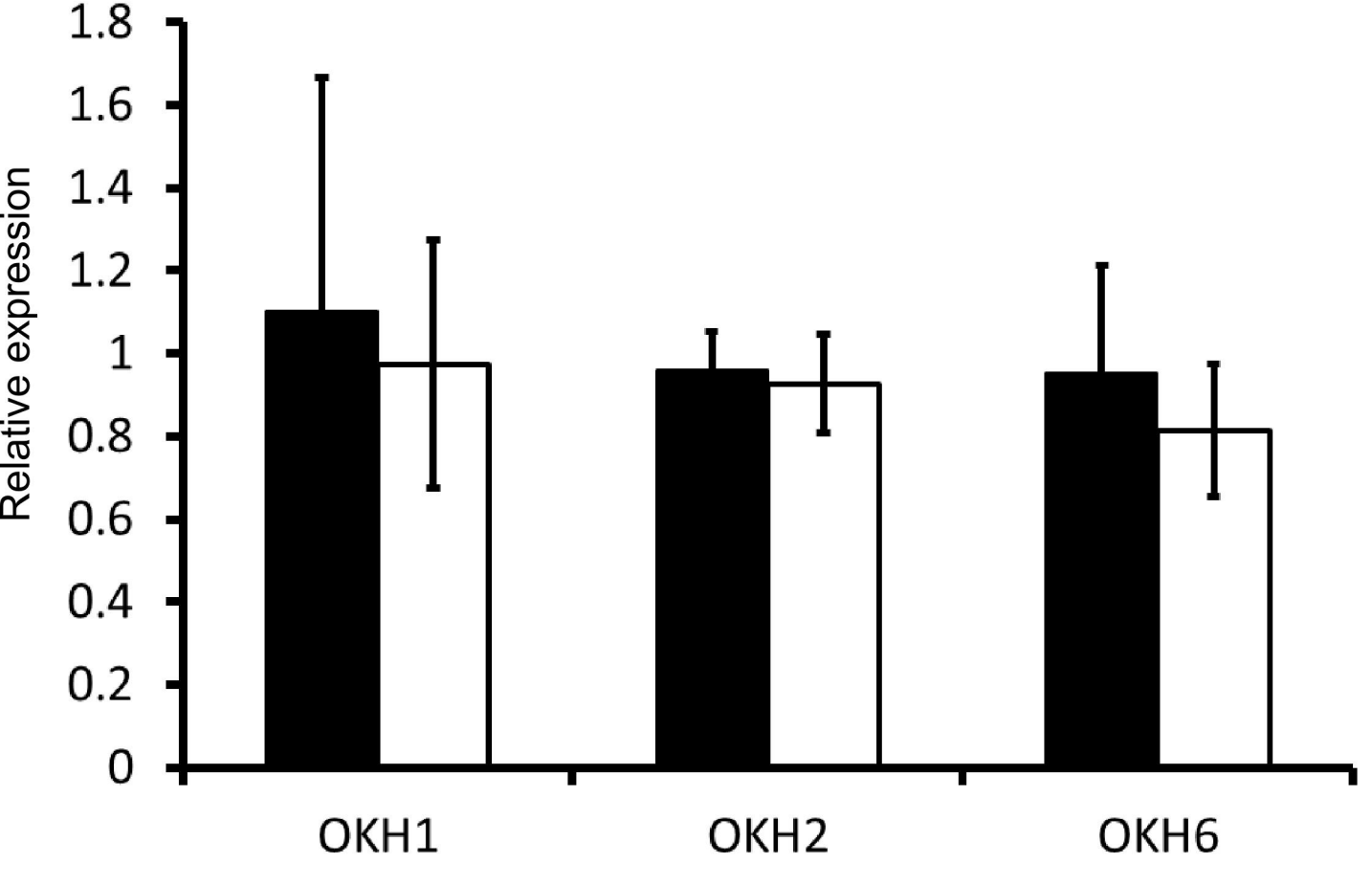

Table1. MIC and MEC against environmental isolates of *A. fumigatus*

Antifungals	No. of isolates										
	Total	With MIC or MEC (μg/mL) of:									
		≤ 0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	> 8
Itraconazole	91					62	26	3				
Voriconazole	91				2	40	46	3				
Amphothericin B	91					5	60	23	3			
Micafungin	91	91										

Table2. MIC and MEC against clinical isolates of *A. fumigatus*

Antifungals	No. of isolates											
	Total	With MIC or MEC[a] (μg/mL) of:										
		≤ 0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	> 8
Itraconazole	22					7	12	3				
Voriconazole	22					6	13	1	1		1	
Amphothericin B	22					2	10	9	1			
Micafungin	22	21	1									

[a] MEC are shown for micafungin.

Supplementary Table 1. Soil samples used in this study.

Sample name	Isolated strains	Produce in the farmland	Azole fungicide used to the farmland
Flower bed	12		Unused
SugarBeet-1	13	Sugar Beet	Tetraconazole
SugarBeet-2	21	Sugar Beet	Tetraconazole
SugarBeet-3	5	Sugar Beet	Tetraconazole
SugarBeet-4	1	Sugar Beet	Tetraconazole
Wheat-1	4	Wheat	Unused
Wheat-2	4	Wheat	Unused
Wheat-3	8	Wheat	Unused
Wheat-4	6	Wheat	Tebuconazole
Wheat-5	7	Wheat	Tebuconazole
Wheat-6	10	Wheat	Unused

† At the sampling, the farmland was during fallow for one growing season.

Supplementary Table 2. Clinical isolates collected in this study.

Isolated strain	Sample	Underlying diseases	Type of aspergillosis
OKH1	Sputum	Abdominal injury	
OKH2	Sputum	Allergic rhinitis, asthma, past history of acute eosinophilic pneumonia	ABPA
OKH6	Sputum	RA, CVD-IP	
OKH8	Sputum	Scleroderma, pneumothorax, CVD-IP	
OKH9	Sputum	Pulmonary tuberculosis, hypertension, AD	SPA
OKH10	Sputum	ITP, heart failure, hypertension	
OKH11	Sputum	Multiple myeloma, hepatitis C	
OKH12[a]	Sputum	Esophageal cancer, lung metastasis, COPD	SPA, CPPA
OKH13	Bronchial blushing	CPFE, DM	SPA
OKH14[a]	Sputum	Esophageal cancer, lung metastasis, COPD	SPA, CPPA
OKH15[a]	Sputum	Esophageal cancer, lung metastasis, COPD	SPA, CPPA
OKH16[b]	Pleural fluid	Lung adenocarcinoma, Hashimoto's disease, asthma	
OKH17[b]	Pleural fluid	Lung adenocarcinoma, Hashimoto's disease, asthma	
OKH21	Sputum	Angioimmunoblastic T-cell lymphoma, aspiration pneumonia	
OKH23	Otorrhea	Allergic rhinitis	Otomycosis
OKH24[c]	Sputum	NTM infection, DM	SPA
OKH25[c]	Tracheal aspiration	NTM infection, DM	SPA
OKH29	Tracheal aspiration	Aspiration pneumonia, cerebral hypoxia	
OKH30	Wound	Burn, heat stroke, higher brain dysfunction	
OKH31[d]	Sputum	CPFE, lung adenocarcinoma (recurrence), ANCA-associated glomerulonephritis, chronic renal failure, CMV pneumonia	CPPA
OKH32	Sputum	Sweet's syndrome, asthma, hypertension, plaque psoriasis	
OKH33	Sputum	AD, prostate cancer, intracerebral hemorrhage	
OKH34[d]	Sputum	CPFE, lung adenocarcinoma (recurrence), ANCA-associated glomerulonephritis, chronic renal failure, CMV pneumonia	CPPA

Abbreviations: RA: rheumatoid arthritis, CVD-IP: collagen vascular disease associated interstitial pneumonia, AD: Alzheimer-type dementia, ITP: idiopathic thrombocytopenic purpura, COPD: chronic obstructive pulmonary disease, CPFE: combined pulmonary fibrosis and emphysema, DM: diabetes mellitus, NTM: nontuberculous mycobacterial, ANCA: anti-neutrophil cytoplasmic antibody, CMV: cytomegalovirus, ABPA: allergic bronchopulmonary aspergillosis, SPA: simple pulmonary aspergilloma, CPPA: chronic progressive pulmonary aspergillosis

Superscripts (a, b, and c) indicates that these strains were isolated serially from each identical patient.

Supplementary Table 3. Primers used in this study.

Primer name	Sequence (5' to 3')	References and Notes
ITS1	TCCGTAGGTGAACCTGCGG	Used to amplify and sequence ITS region [1]
ITS4	TCCTCCGCTTATTGATATGC	Used to amplify and sequence ITS region [1]
ITS5	GGAAGTAAAAGTCGTAACAAGG	Used to amplify and sequence ITS region [1]
NL1	GCATATCAATAAGCGGAGGAAAAG	Used to amplify and sequence D1/D2 region [2]
NL4	GGTCCGTGTTTCAAGACGG	Used to amplify and sequence D1/D2 region [2]
BenA1	AATAGGTGCCGCTTTCTGG	Used to amplify and sequence partial β-tubulin gene of *A. fumigatus* [3]
BenA2	AGTTGTCGGGACGGAAGAG	Used to amplify and sequence partial β-tubulin gene of *A. fumigatus* [3]
rodA1	GCTGGCAATGGTGTTGGCAA	Used to amplify and sequence partial hydrophobin gene of *A. fumigatus* [3]
rodA2	AGGGCAATGCAAGGAAGACC	Used to amplify and sequence partial hydrophobin gene of *A. fumigatus* [3]
cmd5	CCGAGTACAAGGAGGCCTTC	Used to amplify and sequence partial calmodulin gene of *A. fumigatus* [4]
cmd6	CCGATAGAGGTCATAACGTGG	Used to amplify and sequence partial calmodulin gene of *A. fumigatus* [4]
cyp51Aup-F	GAATATATACGTCGATCTGTGTGAC	Used to amplify and sequence *cyp51A* gene of *A. fumigatus*.
cyp51Adown-R	ATCCCAGCAGATACGCTGGTCTCTGC	Used to amplify and sequence *cyp51A* gene of *A. fumigatus*.
cyp51Aup-F	ACAGAATACTGGGCAGCGGGCTGGAG	Used to sequence *cyp51A* gene of *A. fumigatus*.
cyp51A-F	ATGGTGCCGATGCTATGGCTTACGG	Used to sequence *cyp51A* gene of *A. fumigatus*.
cyp51A-F2	TTAGAGTCTCATGTGCCACTTATTGAGAAGG	Used to sequence *cyp51A* gene of *A. fumigatus*.
cyp51A-F3	CTCACAGCCAAAAGTCCTCGAAGAGC	Used to sequence *cyp51A* gene of *A. fumigatus*.
cyp51A-F4	TTTTCAACGTGGATGGAAAGAAAGGAGTCC	Used to sequence *cyp51A* gene of *A. fumigatus*.
cyp51Adown-R2	ACTATCAAAAACAGGTTTTCGCACGAGC	Used to sequence *cyp51A* gene of *A. fumigatus*.
cyp51AFrc	CCGTAAGCCATAGCATCGGCACCAT	Used to amplify and sequence *cyp51A* gene of *A. fumigatus*.
cyp51Aup-F2rc	CTCCAGCCCGCTGCCCAGTATTCTGT	Used to amplify and sequence *cyp51A* gene of *A. fumigatus*.
Afu_cyp51a-rtF	ACAGAACCGCCAATGGTCTT	Used to quantify the expression of *cyp51A* in *A. fumigatus*.
Afu_cyp51a-rtR	CGCCATACTTTTCTCTGCACG	Used to quantify the expression of *cyp51A* in *A. fumigatus*.
cyp51Bup-F	TATCCAGCAAAGTGTGGCCCCGCCAG	Used to amplify and sequence *cyp51B* gene of *A. fumigatus*.
cyp51Bdown-R	TCGTGATATGAAAAAGCACGCCAGC	Used to amplify and sequence *cyp51B* gene of *A. fumigatus*.
cyp51Bup-F2	TTTGTGTCTTCCAGTTTGCTTGGATCC	Used to sequence *cyp51B* gene of *A. fumigatus*.

cyp51B-F	ATGGGTCTCATCGCGTTCATTCTCG	Used to sequence *cyp51B* gene of *A. fumigatus*.
cyp51B-F2	AGGTAAGCGACTTTGGCAGAAACAC	Used to sequence *cyp51B* gene of *A. fumigatus*.
cyp51B-F3	ATGATGATTGCCTTGTTGATGGC	Used to sequence *cyp51B* gene of *A. fumigatus*.
cyp51B-F4	GCAATTTGCATATCTTCAGCTTGGC	Used to sequence *cyp51B* gene of *A. fumigatus*.
cyp51Bdown-R2	CTACGGCCGGTTTCTTTTCTACTATAGGG	Used to amplify and sequence *cyp51B* gene of *A. fumigatus*.
cyp51B-MR	GCCATCAACAAGGCAATCATCAT	Used to sequence *cyp51B* gene of *A. fumigatus*.
Afu_cyp51b-rtF	GACTGCCGCGCAAAGTATG	Used to quantify the expression of *cyp51B* in *A. fumigatus*.
Afu_cyp51b-rtR	GCAGCTTGCCGTTTAGGATG	Used to quantify the expression of *cyp51B* in *A. fumigatus*.

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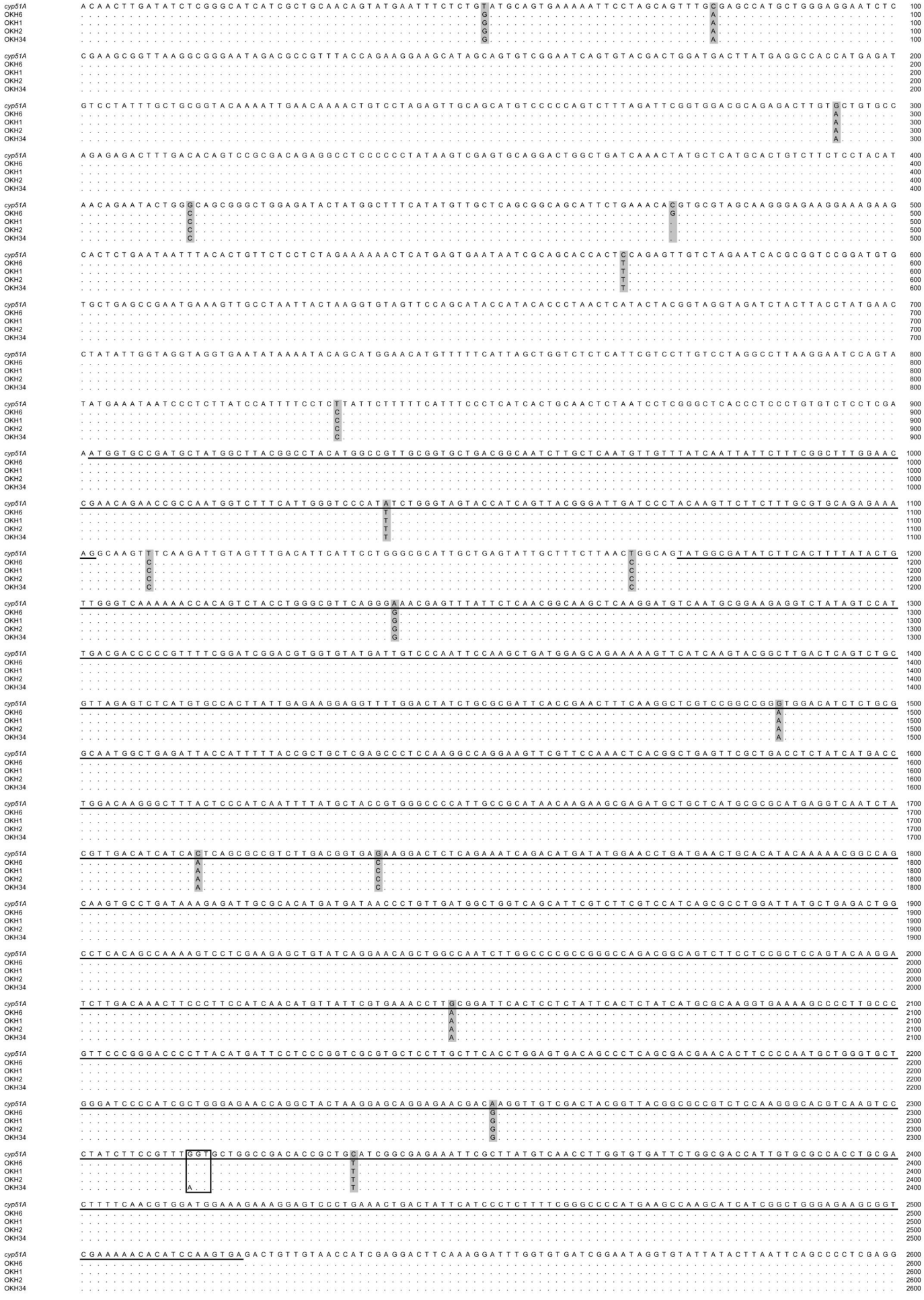
Supplementary Figure 1a



Supplementary Figure 1b