A MALDI-TOF MS-based discriminant model to distinguish azole-resistant Aspergillus fumigatus strains

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Abstract

Aspergillus fumigatus is the major causative agent of aspergillosis in immunosuppressed hosts and patients with chronic pulmonary diseases. Recently, azole resistance of this fungus has become a problem worldwide; thus, rapid detection of resistant strains is desirable in clinical practice. In this study, we established a matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) combined with multivariate and discriminant analysis to detect azole-resistant *A. fumigatus* strains. The score plot from a partial least squares discriminant analysis clearly separated resistant and susceptible strains, regardless of the culture period. The discriminant analysis using the support-vector machine algorithm based on a dataset for strains cultured on potato dextrose agar for 3 days showed the best performance, with a correct answer rate of 84.75–99.85%. We found a MS peak (4359.5 *m/z*) specific to some azole-resistant strains with a 34-bp tandem repeat in the promoter region of *cyp51A*. These tandem repeat strains are extremely rare in Japan. Instead, most resistant strains in Japan have point mutations in *cyp51A*. No specific biomarker for strains with point mutations was found, but it was possible to detect these resistant strains using a model for point-mutation strains.

Introduction

Aspergillus fumigatus is the most frequent cause of aspergillosis in immunosuppressed patients. Azole drugs are used to treat this mycosis, but azole-resistant strains occur worldwide[1]. Rapid identification of the fungal species and its susceptibility to azoles is crucial for the successful treatment of affected patients. *Aspergillus* species are identified on the basis of morphological features and the sequences of genes encoding calmodulin and/or β-tubulin, and both methods require considerable expertise[2]. Azole susceptibility is generally determined by liquid dilution in accordance with standardized protocols. Several easy methods have been proposed to detect point mutations in the *cyp51A* gene, such as surveyor nuclease assay[3], and polymerase chain reaction (PCR) assay[4,5]. However these methods are not widely used in clinical microbiology laboratories.

In the last decade, matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) has become popular as a highly reliable, fast, and easy tool for the rapid identification of clinical microorganisms. This technique is based on the detection of proteins from target organisms with subsequent comparison of their mass spectral peaks against those in an existing database (fingerprinting method). For fingerprinting, mass spectra are generated from repeated measurements, selected peaks are ordered in terms of their frequency of detection and relative intensity, and then the data are aggregated at the strain- or species-level. Although it is more difficult to apply this method to filamentous fungi than to bacteria and yeasts, improved extraction methods and data libraries are available[6]. Several studies have reported on the detection of drug-resistant fungi using proteomic methods[7–10]. More recently, Zvezdanova et al.[11] demonstrated the effectiveness of multivariate analysis and machine learning for the detection of azole-resistant *A. fumigatus* using MALDI-TOF MS data. They showed that machine learning enables accurate identification among closely related species of *A. fumigatus*, and they were able to detect azole-resistant strains with 91.5% accuracy.

The aim of this study was to establish a new discriminant model to identify azole-resistant strains based on a multivariate analysis after MALDI-TOF MS analysis. The experimental procedures were developed considering ease of use, using only common solid media without exposure to antifungal agents. Discriminant models were constructed using data obtained with two widely used instruments, and each model was verified. This new experimental system makes it possible to provide a one-stop analysis to identify each fungal species and predict its drug susceptibility.

Results

Species identification

The percentages of strains correctly identified as *A. fumigatus* in each cultivation period and medium are shown in Fig. 1. In the Microflex analyses (*n*=60, Fig. 1A), the longer the culture time, the lower the matching rate with the database. The day 3 cultures had the highest rates of correct identification with high scores. In the SARAMIS analyses, more than 88.5% of strains were accurately identified from day 10 cultures (*n*=34, Fig. 1B). We tested four types of cultures: two liquid cultures (potato dextrose broth, PDB, and Sabouraud's dextrose broth, SDB) and two solid cultures (potato dextrose agar, PDA, and Sabouraud's dextrose agar, SDA). In the cultures incubated for 1 day at 30°C on PDB, all strains were correctly identified with good scores using the Microflex system (see Fig. S1, supplemental materials).

Validation of the discriminant models

Partial least squares discriminant analyses (PLS-DA) revealed clearer differences between resistant and susceptible clusters in day 3 cultures than in day 5 cultures, and differences between resistant and susceptible clusters from cultures incubated for shorter periods, based on data obtained using both types of machine (Fig. 2, Fig. S2, supplemental materials). Discrimination between azole-resistant and azole-susceptible strains was less accurate for cultures in/on other media, especially SDA, and for cultures in SDB incubated at higher temperatures (to accelerate growth) for more than 24 h. For the culture on SDA (25°C, 3 days), the differentiation between azole-resistant and azole-susceptible strains was not clear (Fig. S2, supplemental materials). Among all the tested datasets, the data from mycelia incubated on PDA at 25°C for 3 days showed the clearest differences between azole-resistant and azole-susceptible clusters. Therefore, we used this dataset for subsequent experiments.

The results of validation of the discriminant models are shown in Table 1. For each strain, the result was based on positive identification six times out of eight replications. The confidence level automatically calculated by the software was averaged. Because a limited number of strains was used in the VITEK test, the training data were also used for validation. Consequently, the correct answer rate was very high (97.06%–100%) as calculated using the random forest (RF) algorithm. The correct answer rate was lower when using the Microflex system, but these values were more realistic because these analyses were based on verification data. The correct answer rate for 3-day cultures on both PDA and SDA was 80.00%–92.50%, or slightly higher for SDA when the RF algorithm was applied instead of the support vector machine (SVM) algorithm. The confidence level of the correct answer was 70% or higher, but in some cases, the confidence level of the wrong answer was also 70% or higher.

Signature peaks of azole-resistant strains

We tested several methods to find peaks that characterize azole-resistant strains. One was to identify peaks that differed between sensitive and resistant strains isolated from the same patient. Data from three patients were compared. Several peaks with significant differences were found in the dataset of each patient (based on a PLS-DA loading plot, $p < 0.005$ in a *t*-test), but none were stably detected in all three patients, and there was no tendency of higher or lower peak intensity (Table S1, supplemental materials). For example, the peak at 3129 *w/z*, located at the edge of the loading plot, was specific to susceptible strains in the VITEK dataset, but specific to resistant strains in the Microflex dataset. Thus, it was impossible to distinguish resistant and susceptible strains on the basis of one or several signature peaks (i.e., biomarkers).

In the PLS-DA score plot combined with an ANOVA test for resistant strains with *cyp51A* mutations, those with the TR motif (a repeated sequence in the *cyp51A* promoter) and those with point mutations in the *cyp51A* sequence formed independent clusters (Fig. 3). There were no discernible subclusters within the cluster of strains of point mutations in *cyp51A*. A similar analyses based on mutations in *hmg1* and *erg6* was conceivable, but was not conducted due to inadequate sample size. Six peaks differed between the TR_{34}/L98H group and the group with point mutations in *cyp51A* (Table 2). In particular, a peak around 4359.5 *m/z* was specific to the TR_{34}/L98H group and was confirmed by MS (Fig. 4).

Discussion

The proteome of *A. fumigatus* varies significantly depending on the incubation period[16], especially after producing conidia (after about 3 days). Aside from conidia, primary and secondary metabolites can also affect protein composition. As shown in this study, the incubation period greatly affected the accuracy of identification by the fingerprint method based on averaged spectra (such as MSP). We focused on determining which conditions were suitable for discriminant analysis given the variability of the data. We found that young hyphae from 3-day cultures were the best materials for the determination of drug resistance and for the fingerprint determination method. Analyses by VITEK MS and the RF algorithm seemed to be more accurate for 10-day cultures, but this has not yet been verified using validation data. Because we used two machines, Microflex and VITEK MS, with very different analytical parameters, the output peak matrices were quite different even for the same strain cultured under the same conditions. Considering future use, we used the default values for analyses as much as possible, because these tend to be used, or are fixed as, the defaults in clinical laboratories. The discriminant analysis models generated using data from each machine showed high accuracy (80–97.06%) (Table 1).

The point mutations in *cyp51A* result in one to several amino acid substitutions, but it is unlikely that the slight amino acid substitutions affect the detection of the protein by MALDI-TOF MS. Most of the resistant strains used in this study had point

mutation(s) in *cyp51A*, whereas TR mutants accounted for more than half (26/40) of the resistant strains in a Spanish study[11]. The accuracy of the discriminant models in this study was similar to, and as high as, those in the Spanish study[11]. Different point mutations did not affect separation on the PLS-DA score plot (Fig. 3). Thus, the resistant strains could be identified regardless of the specific nature of their genetic mutations.

The TR is a repeating base sequence of 34 bp or 46 bp in the promoter region, and it has been reported that its presence increases *Cyp51A* expression[17–19]. Although it has not been determined whether the TR-specific peak is derived from cyp51A, the MS profiles of these strains differed from those of other strains, and they formed a cluster separate from the strains with point mutations. Most of the TR strains are strongly resistant to multiple azoles (all four TR strains used in this study are multi-azole resistant, and three have MICs of 8 μg/mL for itraconazole (ITCZ) and voriconazole (VRCZ)). Therefore, early detection of multi-azole-resistant and highly resistant strains based on a TR-specific peak may be possible. Vermeulen et al.[8] detected proteomic differences between resistant TR strains and susceptible strains in the higher molecular range, as well as candidate biomarker proteins and peptides.

The peak detected in our study (4359.5 *m/z*) corresponded to neither cyp51A nor a ribosomal protein, according to previous studies[8,20]. In addition, the peak was inconsistent with all five peaks reported to be characteristic of cyp51A by Zvezdanova et al[11]. This may be due to differences in locality (Europe *vs.* Asia), culture conditions, and parameters used for measurement and statistical analyses. Interestingly, the peak around 4359.5 *m/z* characteristic of TR_{34}/L98H was shifted to a lower molecular weight in TR_{46}/Y121F/T289A. It may be a degradation product or a modified protein, but further analyses (e.g. tandem mass spectrometry, LC-MS/MS) are required to positively identify this peptide.

Because fungal diseases are treated over a long period of time, it is important to know whether the strain affecting the patient has changed to drug resistant. At present, it takes 7 days or longer to obtain enough conidia for analysis, and then another 72 h to conduct a drug susceptibility test—a total of 10 days or more. DNA-based analyses, either loop-mediated isothermal amplification (LAMP) or sequencing, can be completed within a day after pre-culturing. In this study, the resistant strains were determined based on MICs ≧ 2 μg/mL, but it is expected that the model's accuracy will improve if the dataset is based on MICs ≧ 4 μg/mL. Although the accuracy of the models was not 100%, an identification based on a 3-day culture and the MALDI discrimination model can give a time advantage of a few days. These results combined with those of MIC or genetic tests can be used to develop appropriate treatments, for example, the selection of appropriate drugs, to successfully treat patients.

Methods

Fungal Strains

Sixty strains that were isolated in Japan and preserved at the Medical Mycology Research Center, Chiba University, Japan, though the National Bio-Resource Project (NBRP) were used as the training set of pathogenic eukaryotic microbes in this study. They were clearly identified as *A. fumigatus* sensu stricto on the basis of their *β-tubulin* sequence, and included strains with and without mutations in the *cyp51A*, *hmg1*, and *erg6* genes that are a concern for resistance to azoles[3,12-14]. An additional 40 strains (20 resistant and 20 susceptible strains of different lineages from those of the training set) were used to validate the discriminant models (Table S2, supplemental materials). All strains were confirmed to be susceptible to amphotericin B (AMPH), ITCZ, and VRCZ according to the Clinical and Laboratory Standards Institute reference method for broth microdilution (M38, 3rd edition), and the dried plate method for antifungal susceptibility testing (Eiken Chemicals, Tokyo, Japan). Strains with a MIC of 2 μg/mL or higher for ITCZ or VRCZ were considered as azole resistant.

Culture conditions

Strains were pre-incubated on potato dextrose agar (PDA; Nissui Pharmaceutical, Tokyo, Japan) slants at 25±2 °C, in the dark, for more than 5 days. Several media were used to culture strains before subsequent MALDI-TOF MS analyses: potato dextrose broth (PDB; BD Difco, Franklin Lake, NJ, USA), PDA, Sabouraud's dextrose broth (SDB; DAIGO, Nissui Pharmaceutical, Tokyo, Japan), and SDB containing agar (SDA). Conidia or a small amount of mycelia scraped from a pre-incubated slant were inoculated into or onto these media. Liquid cultures consisted of 5 mL media in 15-mL conical tubes shaken at 9 to 10 rpm on a rotator (Tube Rotator ATR280, As One Ltd., Osaka, Japan) at 25±2 °C in the dark. For solid cultures, the conditions were as described previously[15]. Cultures were incubated for 12 days and sampled on days 1, 3, 5, 7, 10, and 12.

Protein extraction

Each colony of interest growing on the surface of the solid culture medium was swabbed with a sterilized wet cotton plug. The collected mycelia were suspended in 300 µL distilled water and 900 µL ethanol. The mass spectra patterns are known to differ significantly between the center and edges of colonies, so the sampling area was a rectangle (1 × 3 cm area) from the center to the edge of the colony. For liquid cultures, mycelia were collected, centrifuged, washed with distilled water, then centrifuged and washed again with distilled water. Ethanol was added to a final concentration of approximately 80%. In both cases, the samples were treated using the ethanol-formic acid method as per the manufacturer's protocol. Samples were vortexed and centrifuged at 13,000 *g* for 2 min. The pellet was dried for a few minutes, but not to complete dryness as samples that were too dry did not dissolve in the next step. The pellet was then suspended in 20 to 40 µL (depending on the volume of pellet) fresh 70% formic acid by vortexing for about 1 min, and then an equal volume of acetonitrile was added. The suspension was vortexed for more than 1 min and centrifuged at 13,000 *g* for 2 min. Next, 1 µL of the supernatant was spotted into each well of the MALDI-TOF target plate. Once the spot was dried, 1 µL matrix: α-cyano-4-hydroxycinnamic acid (HCCA, Bruker Daltonics, Bremen, Germany; or VITEK MS-CHCA, bioMérieux SA, Lyon, France), prepared following the manufacturer's protocol, was added and allowed to evaporate until crystals formed.

MALDI-TOF MS identification

Two different widely available machines were used for mass spectra analyses. The first was a Microflex LT MALDI-TOF spectrometer (Bruker Daltonics) with MBT Compass 4.1 software and the database MBT Filamentous Fungi Library v. 4.0 (856 MSPs). This machine was automatically calibrated against the Bruker Bacteria Standard. The second machine was the VITEK MS plus (bioMérieux) with the SARAMIS Knowledge Base v.4.14 database. This machine was calibrated using *E. coli* strain ATCC 8739 in accordance with the procedure in the manual. For both machines, measurements were carried out with a mass-to-charge ratio (*m/z*) range from 2,000 to 20,000. The default settings were used for peak detection and the number of laser irradiations, and the standard working procedure as described in the manuals was followed. The measurements were repeated eight times per sample (four spots, each analyzed in duplicate).

Peak analysis

To differentiate between susceptible and resistant strains of *A. fumigatus*, a multivariate analysis was conducted with eMSTAT solution v.1.0.0 (Shimadzu, Kyoto, Japan). The peak lists exported from VITEK MS were used directly. For the Microflex analyses, peak matrixes were generated using the software flexAnalysis 3.4, with the following parameter settings; signal to noise threshold: 3, maximal number of peaks: 100, peak width: 5; and height: 80. These data were exported as a text file. The export parameters meant that when the waveform was converted, arbitrary numbers (in the present study, 100) of peaks were selected in order of the highest frequency and intensity. Partial least squares discriminant analysis (PLS-DA) was conducted with the following settings; *t*-test: $p < 0.005$, multiple comparison: Bonferroni, peak preprocessing normalize: total ion count, internal standard: 2000 Da, threshold: 0.0001%, *m/z* tolerance: 800 ppm, Pareto scaling. Peaks identified as significant in a *t*-test ($p < 0.005$) were used in further analyses.

Establishment of discriminant model

Supervised linear support vector (SVM) and random forest (RF) classification algorithms were applied to the training dataset, using the parameters shown in Table S3. The software automatically performed a self-discriminant analysis with the training dataset. We verified the discriminant analysis model for each culture condition using the validation dataset for different strains obtained using the Microflex system.

Declarations

Data availability

All data generated or analyzed during this study are included in this article and its additional file.

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Author contributions

Investigation, K. Y., E. M., T. K., T. A., K. S., J. I. and S. B.; methodology, validation, K. Y. and S. B.; writing—original draft preparation, S. B. and K. Y.; writing—review and editing, T. A., T. Y. and A. W.; funding acquisition, none. All authors have read and agreed to the published version of the manuscript.

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Competing interests

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Tables

Table 1

Correct answer rate as predicted using discriminant model for strains cultured under different conditions and analyzed using two different MALDI machines (Microflex and VITEK).

training data		algorithm	query data (n)		n. of correct answer	correct rate (%)	Mean CL**	Total (%)
culture and detection	n. of peaks*							
25°C, 3d, PDA, VITEK MS	66	SVM	training data	Resistant (27)	27	100	99.85	82.35
				Susceptible (7)	1	14.29	95.13	
		RF		Resistant (27)	27	100	99.84	97.06
				Susceptible (7)	6	85.71	100	
25°C, 10d, PDA, VITEK MS	39	SVM	training data	Resistant (27)	8	29.63	99.84	44.12
				Susceptible (7)	7	100	97.56	
		RF		Resistant (27)	27	100	99.83	100
				Susceptible (7)	7	100	100	
25°C, 3d, PDA, Microflex	144	SVM	validation data	Resistant (20)	13	65	85.76	80.00
				Susceptible (20)	19	95	84.75	
		RF		Resistant (20)	14	70	77.56	82.50
				Susceptible (20)	19	95	81.60	
25°C, 3d, SDA, Microflex	94	SVM	validation data	Resistant (20)	13	65	95.92	80.00
				Susceptible (20)	19	95	93.91	
		RF		Resistant (20)	18	90	76.88	92.50
				Susceptible (20)	19	95	82.97	
30°C, 1d, SDB, Microflex	22	SVM	validation data	Resistant (20)	15	75	78.94	65.00
				Susceptible (20)	11	55	83.95	
		RF		Resistant (20)	12	60	79.75	67.50
				Susceptible (20)	15	75	82.22	
35°C, 1d, SDA, Microflex	112	SVM	validation data	Resistant (20)	12	60	89.38	60.00
				Susceptible (20)	12	60	93.54	
		RF		Resistant (20)	12	60	77.78	72.50

	Susceptible (20)	17	85	82.22

* Number of significantly different peaks (*t*-test *p* value < 0.005) between resistant and susceptible peak matrixes.

** Confidence level.

Table 2

List of candidate biomarker peaks for TR_{34}/L98H and frequency of peak occurrence in each *cyp51A* mutant.

m/z	*p* value	TR_{34}/L98H(24)	TR_{46}/Y121F/T289A(8)	F203 (8)	F219 (8)	G54 (56)	G138 (32)	G448 (120)	M220 (32)	P216 (24)
3343.50	6.71×10^{-60}	83.0 % (20)	87.5 % (7)	0 %	0 %	14.3 % (8)	0 %	0.8 % (1)	6.3 % (2)	0 %
4359.04	3.38×10^{-38}	75.0 % (18)	0 %	0 %	0 %	28.6 % (16)	0 %	0 %	0 %	0 %
6056.78	2.62×10^{-6}	33.3 % (8)	0 %	0 %	0 %	3.6 % (2)	0 %	2.5% (3)	0 %	0 %
7058.83	4.16×10^{-49}	100 % (24)	100 % (8)	0 %	0 %	0 %	0 %	12.5 % (15)	25.0 % (8)	0 %
8704.76	1.51×10^{-11}	37.5 % (9)	0 %	0 %	0 %	17.9% (10)	0 %	0 %	0 %	0 %
12161.55	2.12×10^{-40}	100 % (24)	100 % (8)	0 %	0 %	0 %	0 %	8.3 % (10)	25.0 % (8)	0 %

Number of data points is shown in parentheses.

Figures

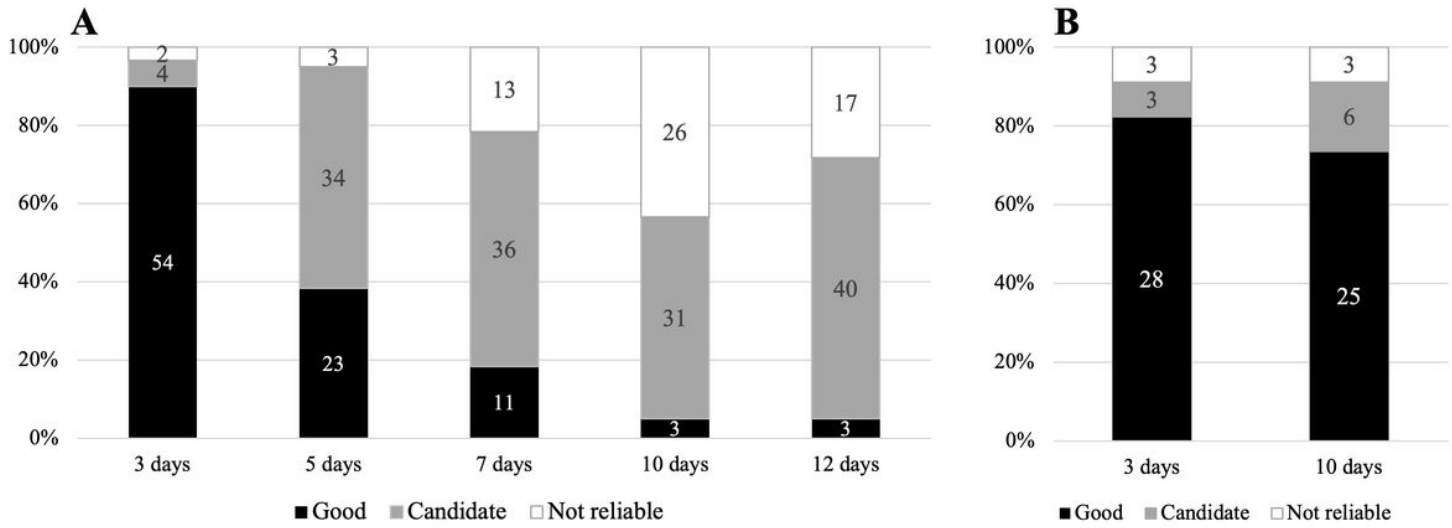

Figure 1

Rate of correct identification of *Aspergillus fumigatus*strains incubated for several days on PDA. **A.** Microflex (*n*=60), **B.**SARAMIS (*n*=34). Good: Correct identification with scores ≧2.00 for Microflex / confidence level (CL) 90.1–99.9 for SARAMIS, Candidate: scores

1.70–1.99 / CL 75.0-90.0, Not reliable: score ≦1.699 / CL ≦74.9.

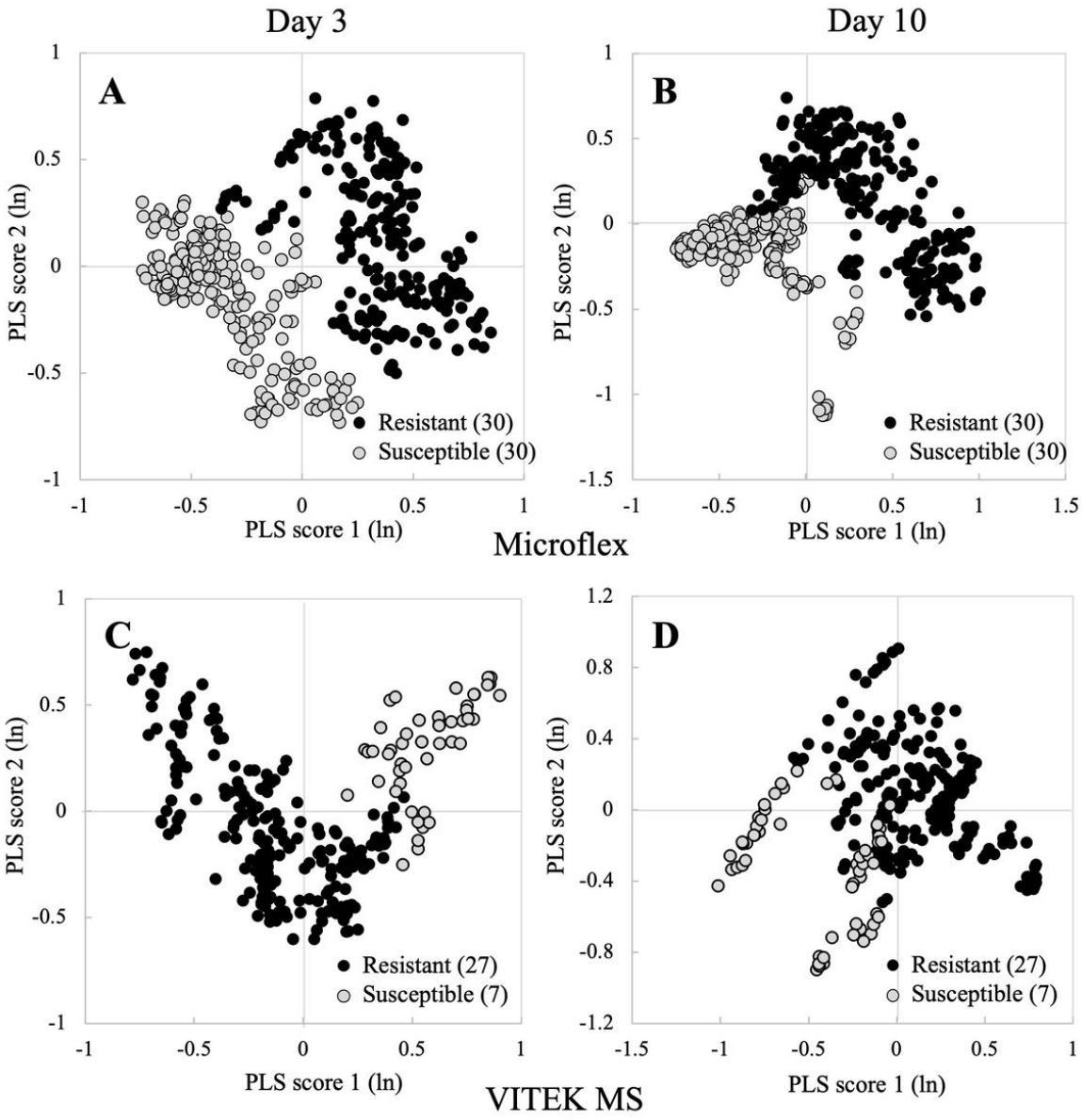

Figure 2

Scatter plots of partial least squares discriminant analysis scores of mass spectra from azole-resistant and susceptible strains of *Aspergillus fumigatus* obtained using two different machines. **A.** Dataset from Microflex, day 3 cultures, **B.** Dataset from Microflex, day 10 cultures, **C.** Dataset from VITEK MS, day 3 cultures, **D.** Dataset from VITEK MS, day 10 cultures.

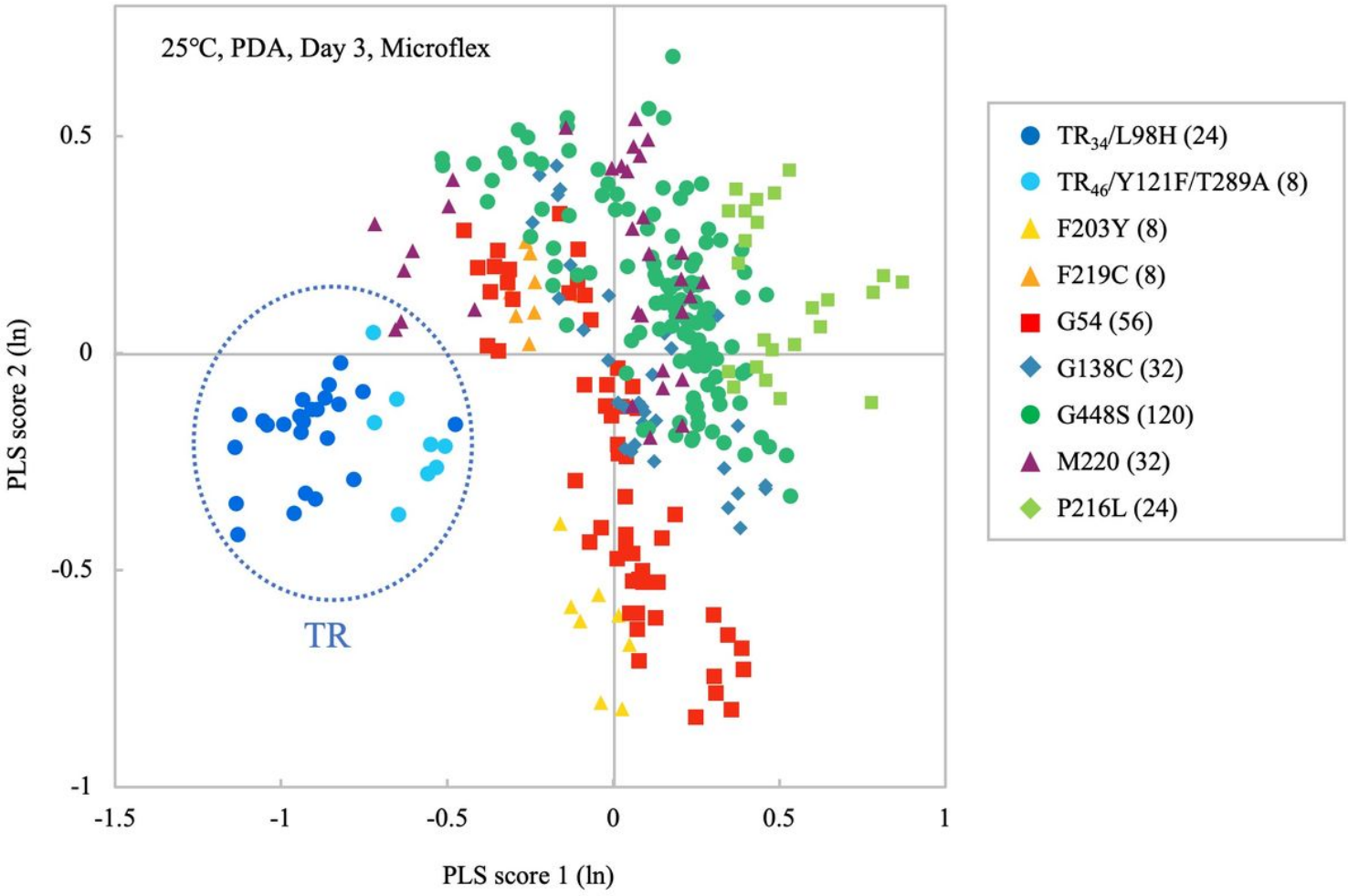

Figure 3

Scatter plots of partial least squares differential analysis scores of mass spectra from strains with different mutations in *cyp51A.*

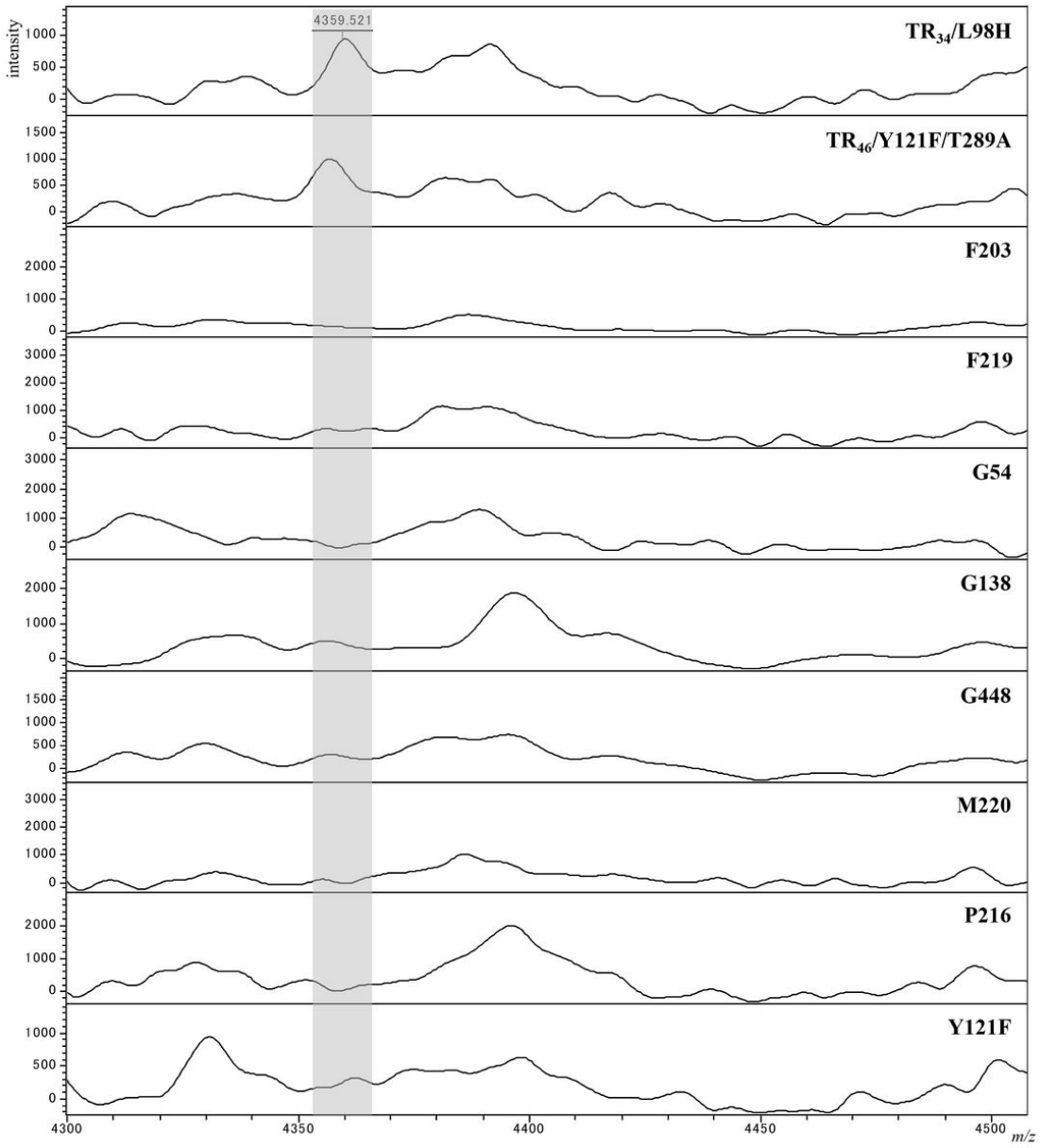

Figure 4

Signature mass peak for azole-resistant TR34/L98H mutant in *cyp51A* (4539.52 *m/z*) (i.e., a biomarker candidate).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFiguresS1andS2.docx
- SupprementaryTablesS1toS3.xlsx