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Interlaboratory Reproducibility of a Single-Locus Sequence-Based Method for Strain Typing of *Aspergillus fumigatus*▽

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Seven international laboratories tested the recently proposed single-locus typing strategy for *Aspergillus fumigatus* subtyping for interlaboratory reproducibility. Comparative sequence analyses of portions of the locus AFUA_3G08990, encoding a putative cell surface protein (denoted CSP), was performed with a panel of *Aspergillus* isolates. Each laboratory followed very different protocols for extraction of DNA, PCR, and sequencing. Results revealed that the CSP typing method was a reproducible and portable strain typing method.

Strain typing of *Aspergillus fumigatus* can be important for detecting outbreaks and in epidemiological investigations. Recently a novel, simple, and rapid single-locus sequence typing strategy was proposed as a typing tool for *A. fumigatus* (2). Genetic diversity in this locus arises from both tandem repeats and point mutations of the gene encoding the putative cell surface protein (CSP), AFUA_3G08990 (2). Balajee et al. employed this method (denoted CSP typing) to subtype 55 epidemiologically linked *A. fumigatus* isolates obtained from six nosocomial outbreaks of invasive aspergillosis and found the technique satisfied the tenets of a good subtyping method (6), since it identified distinct genotypes as well as clusters of closely related isolates (clonal complex). Although a subsequent study found that CSP typing had lesser discriminatory power than a microsatellite-based method, CSP typing remains useful as a quick frontline strategy for *A. fumigatus* strain discrimination (1). Importantly, since CSP typing employs a comparative sequencing strategy, it does not require elaborate training or software for analyses and is relatively user-friendly and economical and therefore amenable for use in reference microbiology laboratories. Other available subtyping methods, such as microsatellite (e.g., Str*Af*)-based assays) (3) and *Afut1* DNA hybridization profiles (*Afut1* method) (4), have superior discriminatory power but need specialized equipment and dedicated software. Also, since reproducibility studies have not been conducted using these techniques, the data obtained cannot be readily shared between laboratories.

Balajee et al. evaluated the CSP typing method for typeability, in vitro stability, intralaboratory reproducibility, and concordance with other typing methods (2). However, the interlaboratory reproducibility of this method has not been tested so far. Given that one of the hallmarks of a good typing method is reproducibility which is independent of the operator, place, and time (5), we examined the reproducibility of CSP typing in diverse laboratory settings with data generated under a wide array of experimental conditions.

To test interlaboratory comparability, a panel of *A. fumigatus* isolates was selected from outbreak isolates whose CSP genotypes were established in a previous study (1, 2). In brief, *A. fumigatus* isolates used in this study were obtained from previous cases in invasive aspergillosis outbreaks and represented both clonal and distinct genotypes (as verified by the CSP typing, *Afut1*, and STR*Af* methods [1, 2]). Species identification of all *A. fumigatus* isolates was confirmed by sequence comparison of the β-tubulin region (2).

The panel consisted of 14 *A. fumigatus* isolates: 5 isolates shared the same CSP type (arbitrarily designated genotype 1), 8 isolates shared another CSP type (genotype 2), and 1 isolate had a unique CSP type (genotype 3). In addition, one isolate of *Aspergillus flavus* (CDC 14) was included as an outlier. Isolates were randomly coded, subcultured on Sabouraud dextrose agar slants, and then sent to seven international laboratories, which represented research, clinical, and reference facilities. Each laboratory was also provided with the following *A. fumigatus*-specific primers: 5′-TTGGGTGGCATTGTGCCAA (forward) and 5′-GGAGGAACAGTGCTGTTGGTGA (reverse). These primers amplify a ~550- to ~700-bp fragment of the AFUA_3G08990 gene (dependent on the number of repeats). The participating laboratories cultured, isolated DNA, and performed PCR, sequencing, and DNA sequence analysis using their own routine methods.

The participating laboratories were requested to do the fol-

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lowing: (i) generate CSP sequences from the panel of isolates and align the sequences using the Af293 (*A. fumigatus* isolate whose genome has been completely sequenced) CSP sequence as a reference (GenBank accession no. XM_749624); (ii) visually identify unique and shared genotypes, (iii) assign arbitrary designations to each distinct CSP genotype represented by one or more isolates in the panel—for example, if isolates 1, 2, and 3 were observed to have related genotypes, they were assigned to genotype X; (iv) submit the arbitrary genotype assignments and all sequences in FASTA format via e-mail to the coordinating laboratory; and (v) send detailed protocols on the methods used to generate the sequences to the coordinating laboratory. Each participating laboratory cultured, isolated DNA, and performed PCR, sequencing, and DNA sequence analysis using methods which were routine in their individual laboratories.

Culture methods included seven different media (both broth and agar based) and two incubation temperatures (30°C and 37°C). For DNA extraction, two laboratories harvested mycelial mats and five harvested mycelia and spores from plates. One laboratory collected only spores for DNA isolation. DNA isolation utilized a variety of methods, including commercially available kits and in-house protocols. Only one laboratory quantitated the isolated genomic DNA and made working dilutions of equal concentrations (10 ng/μl), while the others used the genomic DNA directly in the PCR, regardless of concentration.

PCR cycling was carried out on four models of thermal cyclers from three manufacturers. PCR amplifications were accomplished with either commercially available kits (one laboratory) or in-house PCR mixes (six laboratories), utilizing four different polymerases. All laboratories visualized the PCR products on an agarose gel, either commercial or made inhouse. PCR product cleanup was performed either by use of the ExoSap enzyme reaction (one laboratory), use of magnetic beads (one laboratory), or column purification (five laboratories). Five laboratories estimated the concentrations of purified PCR products by comparison to a commercial mass ladder standard on an agarose gel, while two laboratories utilized the NanoDrop UV reader (Thermo Scientific) to quantitate the PCR products. One laboratory used the PCR products regardless of the concentration. Sequencing was performed using either Applied Biosystems BD 3.1 or BD 1.1 Dye Terminator chemistry or DYEnamic ET Dye terminator chemistry (GE Healthcare) on three different models of capillary electrophoretic sequencers. One laboratory utilized a commercial sequencing service. All laboratories sequenced both the forward and reverse strands, and sequence editing was performed using the Sequence Analyzer, Contig Express, MacVector, Sequencher, or BioEdit software package. Sequence alignments were assembled using the BioEdit 7.0.9, ClustalX 1.83, Lasergene 8.0, or Mega 4.0 software program.

Despite the wide spectrum of reagents, equipment, and methods used to obtain the CSP sequences, five laboratories assigned the correct genotype to all isolates, yielding 100% concordance (Table 1), while laboratories 5 and 6 reported a concordance of only 93% and 85%, respectively. Laboratory 5 reported the sequence from isolate CDC 3 as genotype 1, when the correct designation for this isolate was genotype 2. Similarly, laboratory 6 identified the isolate CDC6 as genotype 2, when the correct designation should have been genotype 3. The sequences obtained by these laboratories were of high quality and were identical to those of the genotypes that were incorrectly assigned. All other sequences generated by these laboratories were also of high quality with no base-call errors. Therefore, we speculate that laboratories 5 and 6 may have reported incorrect genotype designations because of possible cross-contamination with another isolate from the *Aspergillus* panel. Alternatively, this could be also be attributed to an inadvertent exchange of samples that may have occurred at any stage of the process from culturing of the organism to DNA extraction to PCR or sequencing. Six laboratories reported that isolate CDC14 yielded no PCR product; this was expected, since this isolate was *A. flavus* and should not be amplifiable with the primer set provided. Laboratory 6 reported this isolate as belonging to genotype 1, reiterating the likelihood of contamination problems in this laboratory.

TABLE 1. CSP genotypes assigned to the *Aspergillus* panel, as reported by participating laboratories

Isolate	Genotype reported by[a]:						
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7
CDC 1	2	2	2	2	2	2	2
CDC 3	2	2	2	2	1*	2	2
CDC 4	1	1	1	1	1	1	1
CDC 5	2	2	2	2	2	2	2
CDC 6	3	3	3	3	3	2*	3
CDC 7	2	2	2	2	2	2	2
CDC 8	1	1	1	1	1	1	1
CDC 9	1	1	1	1	1	1	1
CDC 10	1	1	1	1	1	1	1
CDC 12	2	2	2	2	2	2	2
CDC 14	NP	NP	NP	NP	NP	1*	NP
CDC 15	2	2	2	2	2	2	2
CDC 19	2	2	2	2	2	2	2
CDC 20	2	2	2	2	2	2	2
CDC 21	1	1	1	1	1	1	1

[a] Each of the laboratories (labs) assigned a genotype number to all *A. fumigatus* isolates (except CDC 14, which is *A. flavus*). NP, no product; *, incorrectly assigned genotype.

The participating laboratories aligned the sequences and assigned genotype scores by visual inspection as described previously (2). The number of isolates in the panel was relatively small, and the differences in repeat number are easy to see in aligned sequences. However, this type of visual analysis would be difficult in larger studies, and a more robust, objective genotype scoring system, which would remove any potential for human error in genotype assignment, should be developed for such analyses. Interestingly, the limiting factor of this typing strategy was strain contamination and/or human error involving sample exchange, rather than sequencing errors or subjective data interpretation. *Aspergillus* spores are easily aerosolized, and extreme care must be taken when working with these organisms to prevent contamination. Assuming that appropriate precautions are taken to prevent contamination, we demonstrate here that CSP typing performed in different laboratories was concordant and results can therefore be compared directly, despite considerable variation in protocols.

Recently the STR*Af* method was demonstrated to have good interlaboratory reproducibility for *A. fumigatus* subtyping (4). In this study, where five laboratories participated, nonspecific

amplification products, bleed-through of the different fluorescent labels, and inexperience of laboratories led to some inconsistencies in results. Here, we present results of another multicenter study for *A. fumigatus* subtyping that also had superior reproducibility. Such multilaboratory reproducibility studies are essential to ensure that any proposed subtyping method can be reliably employed for epidemiological studies.

Additionally and importantly, all data in this study were shared via the Internet, thus confirming that the CSP typing scheme can be a portable and thereby convenient strategy for interlaboratory data sharing or comparison. Furthermore, the data from such studies can easily be stored in a database and archived, retrieved, and reanalyzed at any time, making this a useful tool for global molecular epidemiological investigations of *A. fumigatus*. The use of inexpensive or free Web-based software for data analysis makes this an attractive tool for small or cost-conscious laboratories. In summary, this international, multilaboratory study confirms the reproducibility and portability of the CSP typing method.

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