MYCOLOGY

Multilocus enzyme electrophoresis analysis of *Aspergillus fumigatus* strains isolated from the first clinical sample from patients with invasive aspergillosis

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The genotypes of 50 isolates of *Aspergillus fumigatus* from 11 patients with invasive aspergillosis, obtained from three hospitals in different geographical areas, were determined by multilocus enzyme electrophoresis (MLEE). The study analysed the genetic polymorphism of multiple isolates from the first sample. Seven of the 14 enzymic loci studied were polymorphic, giving rise to eight different electrophoretic types. For nine of 11 patients studied, no polymorphism was observed in isolates within the first clinical sample. Analysis of genetic distance between electrophoretic types demonstrated a genetic heterogeneity within each geographical site. Moreover, some genotypes were preferentially found in a given area and this revealed a population structure within these geographical sites. Therefore, the epidemiology of *A. fumigatus* should be considered separately for each of these areas. The multiple discriminatory markers of MLEE seem to provide a powerful tool for increasing the understanding of the biology of this fungus.

Introduction

Aspergillus fumigatus is an opportunist fungal pathogen that causes high mortality among hospitalised patients [1]. It is one of the most frequent causes of nosocomial pneumonia and the mortality from infection is up to 90% [1]. It affects immunocompromised individuals suffering from severe haematological malignancies [2]. The incidence of infection with this organism has increased over the last 10 years because of the growing number of patients undergoing bone marrow or solid organ transplantation [3], and because of intensive chemotherapy and corticosteroid treatments of malignancies. *A. fumigatus*, commonly isolated from the environment [4], releases numerous conidia that can be inhaled by potential hosts [5]. This may induce allergic asthma, allergic bronchopulmonary aspergillosis, asper-

gilloma, hypersensitivity pneumonitis and invasive aspergillosis.

Epidemiological studies of aspergillosis are essential to understand the biology of this fungus [6], to understand better how it spreads [7] and to improve prevention of aspergillosis [8]. Species identification and phenotype are not sufficient to discriminate between different isolates; however, the development of DNA typing methods has increased the power of discrimination between strains [9, 10]. Random amplified polymorphic DNA (RAPD) [11], restriction fragment length polymorphism (RFLP) analysis, moderately repetitive sequence analysis, sequence-specific DNA primer analysis [12], and restriction endonuclease analysis [13, 14] are routinely used to demonstrate intraspecific diversity in the genus *Aspergillus*. However, protein electrophoretic typing methods, such as immunoblotting and multilocus enzyme electrophoresis (MLEE), have also proved to be useful in various studies. MLEE, first used for *Candida albicans* [15], has provided good results for *Cryptococcus* [16–18] and *Aspergillus* [19] spp. It is a very effective typing

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method, combining both phenotypic and genotypic information.

This study determined the genetic variability at 14 putative loci of 50 isolates of *A. fumigatus* from 11 patients with invasive aspergillosis by means of MLEE. In each case, colonies from the first patient sample were typed by MLEE to investigate the genetic polymorphism of isolates within the same specimen.

Materials and methods

Isolates

The European Research Group on Biotype and Genotype of *Aspergillus* (EBGA) collected *A. fumigatus* clinical isolates at three European hospital centres. This collection was maintained by the Institute of Hygiene and Epidemiological Mycology (IHEM), Brussels, Belgium. Up to five colonies isolated from the first clinical sample, containing at least three colonies, were collected and typed. The study was performed on 50 *A. fumigatus* isolates from 11 patients suffering from haematological malignancies with or without neutropenia. Clinical, radiological and mycological investigations were carried out at three hospital centres (Lyon, France; Grenoble, France; Milan, Italy). Isolates from these hospital centres and IHEM were identified by classical techniques based on conventional morphological identification. The origins of the isolates of *A. fumigatus* are summarised in Table 1.

Preparation of culture lysates

The *A. fumigatus* cultures were grown on a gyratory shaker at 150 rpm in 500-ml flasks containing 150 ml of Sabouraud liquid medium (pH 8) at 37°C for 4 days. The mycelial growth was harvested and washed with 1 M phosphate-buffered saline (PBS). Mycelial fragments were disrupted mechanically at 4°C by the glass

bead method (0.25 mm diameter) for 2 min in a Science Tech MSK homogenizer (B. Braun, Melsungen, Germany). Cell debris was removed by centrifugation at 4000 *g* for 30 min at 0°C. The supernate of each lysate was stored in divided volumes at –20°C before use in MLEE.

MLEE

Starch gel electrophoresis and specific enzyme staining were performed as described previously [15, 16, 19, 20]. In this study, 14 enzymic systems were tested: peptidase 1 (PEP 1, EC 3.4.11, substrate, Val-Leu); peptidase 2 (PEP 2, EC 3.4.11, substrate, Leu-Gly-Gly); peptidase 4 (PEP 4, EC 3.4.11, substrate, Leu-Ala); peptidase 3 (PEP 3, EC 3.4.11, substrate, Phe-Pro); peptidase 5 (PEP 5, EC 3.4.11, substrate, Lys-Leu); glucose phosphate isomerase (GPI, EC 5.3.1.9); malate dehydrogenase (MDH, EC 1.1.1.37); malico-enzyme (ME, EC 1.1.1.40); aspartate-amino-transferase (AAT, EC 2.6.1.1); glucose-6-phosphate dehydrogenase (G₆PD, EC 1.1.1.27); phosphoglucomutase (PGM, EC 2.7.5.1), 6-phosphogluconate dehydrogenase (PGD, EC 1.1.1.43).

Alleles were numbered in decreasing order of their anodal mobility. Each isolate was characterised for the various polymorphic enzyme-encoding loci. Distinct multilocus variants were designated an electrophoretic type (ET).

Calculation of genetic distances

Genetic distances were calculated to quantify genetic divergences between the strains observed by allelic frequencies. Corrected genetic distance (D) is given by the equation:

Table 1. Characteristics of the strains of *A. fumigatus* studied

Patient	Underlying disease	Sample	Date of sampling	Neutropenia	IHEM no.	Origin
A	Lymphoma	BAL	16/11/94	Yes	9378–9382	Lyon
B	Lymphoma	BAL	17/2/95	Yes	9508–9512	Lyon
C	Lymphoma Leukaemia Solid tumour	Sputum	10/1/95	Yes	9447–9451	Lyon
D	Bone marrow transplant	BAL	4/4/95	Yes	9595–9598	Lyon
1	Lymphoma	BA	21/4/94	Yes	9025–9029	Milan
2	Long-term corticosteroid II therapy	Rhinopharyngeal aspirate	24/10/97	Yes	14202–14206	Milan
3	Long-term corticotherapy	BA	9/5/96	No	10054–10056	Milan
α	Lymphoma Bone marrow transplant	BAL	13/1/95	Yes	9418–9420	Grenoble
β	Leukaemia Long-term corticosteroid therapy	BAL	4/10/94	Yes	9347–9351	Grenoble
χ	Lymphoma Kidney transplant	BA	29/5/95	Yes	9720–9724	Grenoble
δ	Leukaemia	BA	18/4/95	Yes	9600–9604	Grenoble

BAL, bronchoalveolar lavage; BA, bronchial aspiration.

$$D^2 = 4^* \frac{\sum_m 1 - (\sqrt{p_{1mi}}\sqrt{p_{2mi}})}{\sum a_m - 1}$$

Where **m** represents the locus number, **p_{nmi}** frequency of allele **i** for locus **m** in the population **n** and **a_m**, the number of alleles at locus **m** [21, 22].

Results

MLEE gave reproducible results; identical results were obtained when isolates were subcultured several times on Sabouraud medium and when electrophoresis was performed on samples from every fourth isolate passage.

Genetic diversity

Seven loci were polymorphic in the 50 strains examined, each with two alleles. The study identified 14 alleles giving eight different ET. The remaining seven profiles (Pep B, Pep C, Pep E, Mdh, Me, Hk, Mpi) were highly conserved. One locus (Pgm) displayed a heterozygous pattern phenotype.

Strain diversity within the same sample Different colonies sampled from the same patient displayed the same multilocus pattern in nine of 11 patients. In two patients the infection was clearly caused by two different strains. For these two patients (patient 1 and patient α), strains differed from the others by one allele at the locus Fum. This difference separates ET3 and ET5 in Milan and ET7 and ET8 in Grenoble (Table 2). In other cases, only one genotype was found for each patient.

Strain diversity inside the same area. There was extensive genetic variability within geographical sites. In Grenoble, three different genotypes were found from four patients studied; pairs of ETs differed by one-to-two loci (Table 3). In Milan, three different genotypes were found from three patients; pairs of ET differed by one-to-three loci (Table 3). In Lyon, three different genotypes were identified from four patients studied and pairs of ET differed by two-to-four loci (Table 3).

Strain diversity between geographical sites. Some genotypes were preferentially found in a given area. ET1, ET7 and ET8 were found only in Grenoble; ET5 and ET6 only in Milan and ET2 and ET4 only in Lyon. Only ET3 was found in both Lyon and Milan. Moreover, GPI 2 allele was found only in strains from Grenoble and Pep1-2 and Pep4-2 alleles were found only in strains from Lyon (Table 2). These results suggest the existence of extensive intra- and inter-site heterogeneity and, thus, a relative independence of each area.

There is a lack of correlation between the genotype of each of the strains studied and the geographic distance. Genetic distances between ET from the two nearest cities (Lyon and Grenoble) were greater than between ET from Lyon and Milan (Table 2). This was shown by differences between pairs of ET (three-to-seven between Lyon and Grenoble and three-to-five between Lyon and Milan) (Table 3).

Discussion

MLEE is one of numerous typing methods developed for the differentiation of *A. fumigatus* isolates. It has a relatively high degree of discriminatory power, but also allows assessment of the structure and differentiation of the sample studied, including its genetic diversity [15, 16, 19]. It was used as the main technique in this study because it is considered to be a useful epidemiological tool for increasing understanding of the biology of *A. fumigatus*.

A genetic analysis of 50 strains of *A. fumigatus* from three geographical areas was performed. Some studies have reported an extensive variation in this fungus. Rinyu *et al.* found that 61 strains could be classified in 19 groups, by analysing only seven enzymic systems [23]. Matsuda *et al.* distinguished 14 ETs within 34 strains, with only three polymorphic enzymic systems [24]. Rodriguez *et al.* found 48 allelic combinations within 71 isolates [19]. In the present study, the level of diversity observed was less than that reported in the above studies. This was due to the strong homogeneity of samples studied, and is in agreement with studies from Radford *et al.*, who found 11 distinct DNA types among a total of 119 isolates [25]. In the present study, for each patient the specimen chosen for typing was the first sample, containing three-to-five colonies. The study demonstrated that there was a strong homogeneity within the same specimen with strains exhibiting the same multilocus enzymic profile. Only two patients were shown to have an infection caused by two different strains. The difference between these strains was due to only one allele at the locus Fum (Table 2). This might be due to a recent mutation and this result is very important for the investigation of the effect of therapy on these strains, as mutation can interfere with the treatment of infections by some strains. If *A. fumigatus* is considered to be diploid [19], two alleles would be affected by the same mutation, and this type of mutation occurs rarely. However, if *A. fumigatus* is considered to be haploid [26], only one allele can be affected by this mutation. This second possibility is the most likely. The apparently heterozygous pattern at the locus Pgm may be due to a duplication of this gene. However, in most cases, only one strain was responsible for the infection, regardless of the geographical location. The present study focused on human clinical isolates and is in agreement with some other studies [25, 27], which showed that usually only one strain of

Table 2. Allelic profiles* and origin of isolates of *A. fumigatus*

		Alleles at the indicated enzyme locus ^{†‡}														
Patient and IHEM no.	Origin	Pep 1	Pep 2	Pep 3	Pep 4	Pep 5	Aat	Me	Mdh	Hk	Fum	Gpi	Mpi	Pgm	Pgd	ET no.
A 9378	Lyon	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	3
9379		1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	3
9380		1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	3
9381		1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	3
9382		1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	3
B 9508		2/2	1/1	1/1	2/2	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	4
9509		2/2	1/1	1/1	2/2	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	4
9510		2/2	1/1	1/1	2/2	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	4
9511		2/2	1/1	1/1	2/2	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	4
9512		2/2	1/1	1/1	2/2	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	4
C 9447		1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	3
9448		1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	3
9449		1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	3
9450		1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	3
9451		1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	3
D 9595		2/2	1/1	1/1	2/2	1/1	2/2	1/1	1/1	1/1	1/1	1/1	1/1	1/2	1/1	2
9596		2/2	1/1	1/1	2/2	1/1	2/2	1/1	1/1	1/1	1/1	1/1	1/1	1/2	1/1	2
9597		2/2	1/1	1/1	2/2	1/1	2/2	1/1	1/1	1/1	1/1	1/1	1/1	1/2	1/1	2
9598		2/2	1/1	1/1	2/2	1/1	2/2	1/1	1/1	1/1	1/1	1/1	1/1	1/2	1/1	2
1 9025	Milan	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	3
9026		1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	3
9027		1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	2/2	1/1	1/1	1/1	1/1	5
9028		1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	3
9029		1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	3
2 14202		1/1	1/1	1/1	1/1	1/1	2/2	1/1	1/1	1/1	1/1	1/1	1/1	1/2	1/1	6
14203		1/1	1/1	1/1	1/1	1/1	2/2	1/1	1/1	1/1	1/1	1/1	1/1	1/2	1/1	6
14204		1/1	1/1	1/1	1/1	1/1	2/2	1/1	1/1	1/1	1/1	1/1	1/1	1/2	1/1	6
14205		1/1	1/1	1/1	1/1	1/1	2/2	1/1	1/1	1/1	1/1	1/1	1/1	1/2	1/1	6
14206		1/1	1/1	1/1	1/1	1/1	2/2	1/1	1/1	1/1	1/1	1/1	1/1	1/2	1/1	6
3 10054		1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	3
10055		1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	3
10056		1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	3

Table 2. (cont.)

Patient and IHEM no.	Origin	Alleles at the indicated enzyme locus ^{†‡}														ET no.
		Pep 1	Pep 2	Pep 3	Pep 4	Pep 5	Aat	Me	Mdh	Hk	Fum	Gpi	Mpi	Pgm	Pgd	
α 9418	Grenoble	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	2/2	1/1	1/1	2/2	7
9419		1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	2/2	2/2	1/1	1/1	2/2	8
9420		1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	2/2	1/1	1/1	2/2	7
β 9347		1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	2/2	1/1	1/1	1/1	1
9348		1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	2/2	1/1	1/1	1/1	1
9349		1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	2/2	1/1	1/1	1/1	1
9350		1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	2/2	1/1	1/1	1/1	1
9351		1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	2/2	1/1	1/1	1/1	1
χ 9720		1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	2/2	1/1	1/1	1/1	1
9721		1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	2/2	1/1	1/1	1/1	1
9722		1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	2/2	1/1	1/1	1/1	1
9723		1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	2/2	1/1	1/1	1/1	1
9724		1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	2/2	1/1	1/1	1/1	1
δ 9600		1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	2/2	1/1	1/1	1/1	1
9601		1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	2/2	1/1	1/1	1/1	1
9602		1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	2/2	1/1	1/1	1/1	1
9603		1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	2/2	1/1	1/1	1/1	1
9604		1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	2/2	1/1	1/1	1/1	1

*Results of four independent experiments for each enzymic system.

[†]For abbreviations, see *Materials and methods*.[‡]1/2, alleles 1 and 2 presented by heterozygotes.

Table 3. Nei's genetic distances (lower left matrix) and number of loci showing different alleles (upper right matrix) between the different *A. fumigatus* ET analysed

	ET1	ET2	ET3	ET4	ET5	ET6	ET7	ET8
ET1	–	5	1	3	2	3	1	2
ET2	0.370	–	4	2	5	3	6	7
ET3	0.074	0.269	–	2	1	2	2	3
ET4	0.241	0.095	0.154	–	3	4	4	5
ET5	0.154	0.370	0.074	0.241	–	3	3	2
ET6	0.179	0.160	0.095	0.269	0.179	–	4	5
ET7	0.074	0.481	0.154	0.336	0.241	0.269	–	1
ET8	0.154	0.606	0.241	0.442	0.154	0.370	0.074	–

A. fumigatus is responsible for the infection of a particular patient. However, some authors found that some patients may be infected by several different strains in the late stages of the infection [25, 28]. Thus, the best strategy for epidemiological studies to determine the source of an infection is to type several colonies within the same sample.

The present study demonstrated that infection by *A. fumigatus* is very different from that caused by *C. albicans*. Multiple strains of *C. albicans* have been found in the oral cavity of an individual patient in the early stage of infection [29, 30], and only one strain becomes predominant during an infection. In contrast, only one strain of *A. fumigatus* is responsible for the infection in most cases of invasive aspergillosis. Multiple strain genotypes can be found post-mortem, or in cases of lung transplant patients suffering from necrotising aspergillosis, bronchitis aspergillosis, or bronchial colonisation [12]. These data are very important for determining the mechanism of infection of this opportunist fungus and proves that the mechanisms of *A. fumigatus* and *C. albicans* are quite different.

Although the number of strains studied was too small to provide phylogenetic information, the data (Tables 1 and 2) demonstrate the relative heterogeneity between strains within each geographical site. Most ETs are closely related within a sampling site (ET1, ET7, ET8 in Grenoble; ET5, ET6 in Milan; ET2, ET4 in Lyon). Moreover, these data suggest that the global diversity is less extensive within each geographical site than between two different sites. This suggests that strains may be adapted to a particular hospital environment and that the epidemiology of *A. fumigatus* should be studied independently within each geographical area. This situation may be the result of local selective pressure [31], including climate, environmental adaptation and therapeutic selection, coupled with low migration rates of *A. fumigatus* conidia.

MLEE provides a powerful tool for developing a better understanding of the local epidemiology of this fungus. However, these conclusions must be confirmed by other studies, using DNA typing methods with the same strains, to compare the results. These different

techniques may clarify whether it is sufficient to type one isolate from each specimen to determine the strain responsible for invasive aspergillosis.

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