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DNA Typing Suggests Pigeon Droppings as a Source of Pathogenic *Cryptococcus neoformans* Serotype D

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The unusual proportion of serotype D strains of *Cryptococcus neoformans* infecting patients diagnosed with cryptococcosis in some parts of France prompted the analysis of DNA fingerprints obtained with 26 clinical and 29 environmental isolates from the same area. Our results suggest that pigeon droppings are a potential source of pathogenic strains of *C. neoformans* serotype D, as previously demonstrated for serotype A.

Infection due to *Cryptococcus neoformans* appears as a disseminated meningoencephalitis occurring mainly in immunocompromised individuals (12). Cryptococcosis has emerged as the fourth most common lethal infection among AIDS patients and affects 5 to 10% of these patients in Western countries (3). The encapsulated yeast exists in two varieties and four serotypes: serotypes A and D for *C. neoformans* var. *neoformans*, with a worldwide distribution, and serotypes B and C for *C. neoformans* var. *gattii*, limited to tropical and subtropical regions (1, 11, 13). Even though serotypes A and D have been isolated from various sources in nature and associated with pigeon droppings, the ecological niche remains uncertain (1, 18). For *C. neoformans* var. *gattii* serotype B, a specific ecological association with *Eucalyptus calmadulensis* and *Eucalyptus tereticornis* has now been established (7, 15). In addition to differences in the distribution of the varieties, variations in pathogenicity have been reported (14, 17), but no differences within a variety had been described until the publication of a recent report showing that individual and geographical factors were associated with infections due to serotype D (5). Among these factors was a heterogeneous distribution of serotype D infections around France, with a higher percentage in the Southwest. This result suggested a direct influence of the environment on the infecting serotype. It raised the question of whether a genetic similarity between clinical and natural isolates of the D serotype could be identified, as previously done for serotypes A and B (2, 16, 20, 21).

C. neoformans isolates recovered from 26 patients diagnosed with meningitis in the city of Bordeaux, France, from 1991 to 1994 were studied. The majority of patients (74%) were white male human immunodeficiency virus-infected patients (mean age = 40 years). Samples of pigeon excreta (n = 172) were collected from 48 different locations in the city of Bordeaux over a 5-month period (May to September 1993). Sites of collection included roofs and cornices, and soil heavily contaminated with pigeon droppings was also collected. Rehydrated samples were plated on Niger seed agar, and melanin-rich colonies were selected.

All environmental and clinical isolates were identified on the basis of a positive India ink test result, urease production, and sugar assimilation patterns by using commercially available strips (ID 32C; BioMérieux, Marcy-l'Etoile, France). Canavanine-glycine-bromothymol blue medium (CGB), D-proline assimilation, and a direct immunofluorescence assay with a monoclonal antibody were used to identify the variety and the serotype (4). The mating type was determined on nonenriched medium (V-8 juice agar) by crossing all the isolates with reference strains B-4500 (mating type α) and B-4476 (mating type **a**) (both kindly donated by K. J. Kwon-Chung, National Institutes of Health, Bethesda, Md.). *C. neoformans* total DNA was extracted by a previously described method (6). Restriction fragments generated by digesting total-DNA samples to completion with the restriction enzyme *Acc*I (New England BioLabs, Inc., Beverly, Mass.) were separated by electrophoresis through 0.8% agarose gels and transferred onto positively charged nylon membranes (Amersham). The linear plasmid UT-4p was labelled with [^{32}P]dCTP (Amersham) and hybridized to digested DNA (19). Filters were then washed and exposed to X-ray film. Fingerprint pattern analysis was performed by using the software Taxotron developed by P. D. Grimont (Institut Pasteur, Paris, France), which generates a schematic view of the gels. The decision regarding identity or difference was based on the number and the sizes of the major bands. A small shift in mobility of a major band was not considered significant, since we observed during preliminary experiments that DNA purification and electrophoresis itself were able to affect global mobility but not the number of major bands whereas the number of minor bands was altered depending on the duration of the film exposure (data not shown).

C. neoformans was recovered from 10 different locations (21%) and 19 different samples (11%), and 29 colonies were blindly selected for subsequent studies (1 or 2 colonies/positive sample). Overall, 26 clinical isolates (14 serotype A and 12 serotype D, designated by a number prefixed with A or D) and 29 environmental isolates (16 serotype A and 13 serotype D, designated by a number prefixed with E) were studied. A mixture of serotype A and D was found in one sample (E57). As reported earlier (8, 10), mating type α was predominant, with only two mating type **a** strains among clinical isolates of serotype D (Table 1).

Fingerprinting analysis showed more heterogeneity among serotype D (13 different patterns) than among serotype A (9 different patterns) isolates (Fig. 1). The method was discriminatory enough to allow identification of several genotypes within the same environmental sample (isolates E13A, E13B, and A13C in Fig. 1A; isolates E20A, E20B, and E20C in panel

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TABLE 1. Characteristics of the isolates studied

Characteristic	Value for isolate group	
	Clinical (n = 26)	Environmental (n = 29)
No. from human immunodeficiency virus-infected patients	24	
Mean age of patients (yr)	40	
No. of serotype A/D	14/12	16/13
No. of mating type α/**a**	22/2[a,b]	28[b]

[a] Both strains of mating type **a** were discovered among serotype D isolates.
[b] The remaining isolates were sterile.

D) or within the same location (isolates E124A and E125A in panel D).

The fingerprints were more complex with serotype D isolates and were difficult to group, in contrast to the situation with serotype A fingerprints, for which most of the isolates fitted in previously described subgroups (20). Among the French isolates, patterns IV (7 isolates) and VI (15 isolates) were the most common. In the study published by Varma and collaborators, the most common pattern was pattern V, identified for 42 isolates of the 83 clinical and environmental isolates recovered from North America, Africa, and Northern Europe, whereas some genotypes (pattern IV, 7 isolates including 5 from California; pattern VI, 3 African isolates; pattern VII, 1 Californian isolate) were rarely encountered. Patterns IV and VII were predominant among clinical isolates from Japan (9). Taken together, these results suggest that genetically distinct populations of *C. neoformans* are found in different parts of the world. A typing method that could further differentiate isolates based on geographical differences would be of interest to uncover the physiopathology of the infection and to demonstrate whether the infecting isolate is acquired early in life or later on when an immune defect alters the natural defenses.

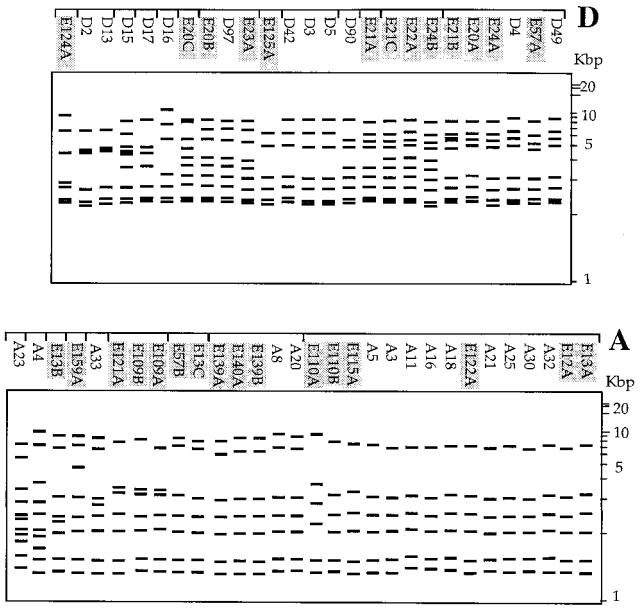

FIG. 1. Schematic representation of the fingerprints obtained with UT-4p on serotype D (top) and A (bottom) isolates. Isolates were recovered from clinical (code numbers starting with A or D) and environmental (shaded code numbers) sources in Bordeaux, France. They are grouped according to the patterns generated with the software Taxotron. For serotype D isolates, only bands above the characteristic doublet (2.1 to 2.3 kbp) were taken into account because bands of smaller molecular size were usually hazy.

Restriction fragment length polymorphism patterns of some clinical and environmental isolates of both serotypes were indistinguishable by this method: D49 and D4 were closely related to E57A, E24A, and E20A, and D90 and E21A had the same pattern, as did D97 and E23A (Fig. 1). For serotype A isolates, pattern VI included isolates coming from clinical (e.g., A32 and A5) and environmental (e.g., E12A and E110B) sources, as did pattern IV, with clinical isolates (A8 and A20) and isolates recovered from pigeon droppings (e.g., E139A and E57B).

Our study design does not allow comparison of the relative percentages of serotype D strains among clinical and environmental isolates, nor does it provide information on the exact proportion of samples that contained a mixture of serotypes, mating types, or genotypes. It clearly shows, however, that pigeon droppings contained a genetically heterogeneous population of *C. neoformans* serotypes A and D in which some isolates are similar to infection-causing organisms. These results do not prove, however, that patients were infected with the corresponding isolates from pigeon excreta, though they are consistent with this hypothesis. In previous studies, genetically related isolates of serotype A were found among clinical and environmental strains, suggesting that pathogenic strains of *C. neoformans* serotype A can be found in the environments of patients at risk for cryptococcosis (2, 9, 20, 21). Our study demonstrates that pigeon droppings can be identified as a potential source of pathogenic strains of serotype D as well.

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