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MYCOLOGY

A rapid method for detecting extracellular proteinase activity in *Cryptococcus neoformans* and a survey of 63 isolates

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A rapid method to detect extracellular proteolytic activity around colonies of *Cryptococcus neoformans* was developed with tannic acid used to complex with residual protein in a solid medium. A survey was conducted with 32 isolates of *C. neoformans* var. *gattii* and 31 isolates of *C. neoformans* var. *neoformans* which were cultured on medium containing gelatin as the sole nitrogen source. The annulus of clearing around fungal colonies was >1.2 mm in 24 (77%) isolates of *C. neoformans* var. *neoformans* compared with only 7 (22%) isolates of *C. neoformans* var. *gattii*. There was no difference in proteolytic activity between environmental and human clinical isolates of *C. neoformans*. However, there was a difference between the size of the annulus around animal isolates of *C. neoformans* var. *neoformans* and isolates of the same variety from other sources. The annuli around the 14 animal isolates were all >1.2 mm, while 7 (70%) of 10 human clinical isolates and only 3 (43%) of 7 environmental isolates were scored in the high proteinase range. A difference between the genetic types (as characterised by RAPD typing) of *C. neoformans* var. *gattii* was also evident with 17 (77%) of 22 VG-I isolates having a small annulus compared with only 1 (17%) of 6 VG-II and VG-III isolates with annuli of similar size. Relatively low proteinase production by *C. neoformans* var. *gattii* may reduce local and systemic spread of infection in mammalian hosts.

Introduction

Cryptococcus neoformans, a pathogenic yeast-like fungus, causes pulmonary and neurological disease. There are two recognised varieties, *C. neoformans* var. *neoformans*, representing serotypes A and D, and *C. neoformans* var. *gattii*, representing serotypes B and C. A recent report argues for the separation of serotype A from *C. neoformans* var. *neoformans* to a new variety, *C. neoformans* var. *grubii*, on the basis of phenotypic and molecular genetic differences [1]. The existing nomenclature has been retained for this report. The recognised varieties differ in ecology, epidemiology and clinical manifestation [2–5]. The likely route of infection is by inhalation of infectious propagules from the environment [6, 7], followed by dissemination to other organs *via* the blood stream. The factors responsible for tissue penetration by cryptococci have not been defined.

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Virulence determinants of *C. neoformans* that have been well characterised to date primarily promote survival of the fungus in the host. For example, the polysaccharide capsule may protect against phagocytosis [8], laccase activity protects against leucocyte oxidants [9, 10] and calcineurin promotes survival at physiological temperatures [11]. Phospholipase (PL) activity may facilitate tissue invasion [12], but this has not been confirmed by characterisation or disruption of the PL gene in either variety of *C. neoformans*.

Proteinases are important virulence determinants in bacteria. By compromising host defences, causing tissue damage or increasing vasodilatation, they may provide nutrients for the pathogen and aid in its dissemination [13, 14]. Amongst the fungi, *Candida albicans* has nine isoforms of secreted aspartyl proteinase (SAP). Each of these is differentially expressed during the infectious process [15]. The loss of SAP1, SAP2 or SAP3 individually [16], or SAP4–SAP6 together [17], results in loss of virulence. Proteinases produced by species of *Aspergillus* are poorly defined as virulence factors. Although variation in the

elastinolytic activity of *A. fumigatus* affects its virulence [18, 19], disruption of any or all of the known extracellular proteinases does not result in loss in virulence [20–22].

Extracellular proteinase activity in *C. neoformans* was first reported in 1972 [23]. Cryptococcal proteinases degrade several host proteins such as elastin, collagen, fibrin, fibrinogen, complement factors and immunoglobulins [23–25], suggesting that they may be important in tissue disruption and perturbation of host immunity. Chen *et al*. [25] partially characterised cryptococcal extracellular proteolytic activity *in vitro* as serine proteinase(s). Purification of the proteinase enzyme(s) has proved difficult, because detection of the activity requires extended periods of incubation in the substrate [25, 26].

Tannic acid is a relatively non-toxic substance that forms an insoluble complex with proteins. It has been used to detect proteins in polyacrylamide gels [27] and in tissue samples after counter-staining with an iron compound [28]. It has also been used to complex proteins in solid media to assess proteinases that remain functional in the presence of tannin [29]. However, some proteinases are destroyed by tannin or are unable to hydrolyse proteins complexed with tannin.

This study compared the use of tannic acid to detect extracellular proteinase activity of *C. neoformans* on solid media with other methods. A survey of 63 *C. neoformans* isolates from environmental and clinical sources was performed to determine the variation in proteinase production by isolates from different sources. A recent survey of proteinase production by this pathogen has been published; however, it only examined proteinases of *C. neoformans* var. *neoformans* [30].

Materials and methods

C. neoformans isolates and culture conditions

Isolates of *C. neoformans* were obtained from environmental and clinical (human and animal) sources and were representative of different genetic types as assessed by a randomly amplified polymorphic DNA (RAPD) method [31–34]. Isolates were cultured from stock and maintained on Sabouraud-2% Dextrose Agar (SAB; Difco).

Proteinase assay

Isolates were cultured on defined medium containing (/L): KH_2PO_4 1.0 g, $MgSO_4$ 0.5 g, NaCl 0.1 g, $CaCl_2.2H_2O$ 0.1 g, $MnSO_4.4H_2O$ 0.4 mg, thiamine.HCl 0.4 mg, $FeCl_3$ 0.2 mg, Na_2MoO_4 0.2 mg. The pH was adjusted to 5.5 with NaOH and Bacto Agar 12 g was added. Approximately 20 ml were poured into 90-mm petri dishes and allowed to set. Defined medium containing a nitrogen source – 10 mM NH_4Cl or skim milk powder (SMP) 0.5–1.0% w/v or gelatin bloom 75 (Sigma) 1.0% w/v or bovine serum albumin (BSA; Sigma) 0.5–1.0% w/v – and a carbon source (galactose, fructose or xylose 1.0% w/v, or glucose 0.5–1.0% w/v) was prepared and *c*. 5 ml were poured on to the base layer of defined medium. Plates were incubated at 30°C in humid conditions, to reduce desiccation, for 2–14 days.

Plates containing gelatin and inoculated with different strains of *C. neoformans* were assessed after 14 days. Residual protein was fixed by flooding the plate with *c*. 5 ml of one of the following agents: $HgCl_2$ 5–10% w/v, $ZnCl_2$ 5–15% w/v, $ZnSO_4$ 10–30% w/v or tannic acid (Sigma) 5–10% w/v. The time of exposure varied with the agent used. The plates were washed thoroughly with de-ionised water. In some experiments isolates were washed off the plates with de-ionised water before fixation for observation of hydrolysis under the colony.

Plates stained with Coomassie Brilliant Blue (CBB), as described by Aoki *et al*. [26], were first fixed with trichloroacetic acid (TCA) 20% v/v for 2 h at room temperature. Colonies were washed off the plate with de-ionised water, as cell debris interfered with the assay. A solution of CBB 0.1% w/v in de-stain solution (methanol 40% v/v, acetic acid 10% v/v) was added and allowed to stand for 30 min. The plates were washed repeatedly in de-stain solution until clearing was visible.

Survey conditions

Isolates were inoculated on to basal medium containing gelatin 1% and galactose 1% from a single colony picked from a SAB agar plate with a 1-μl disposable loop. Plates were incubated at 30°C for 14 days in humid conditions to prevent drying. The width of the annulus (the radius of the colony subtracted from the radius of the zone of clearing) was measured. Preliminary results showed that the annulus produced by colonies of *C. neoformans* was relatively constant regardless of the colony size (data not shown). It was assumed that the size of the annulus correlated with the proteinase activity of *C. neoformans*.

Statistical analysis

The annuli around colonies were measured on two axes. Duplicate colonies were grown on different plates to limit measuring errors. The average annular size of each isolate was tallied for each population and the Mann–Whitney U test, a non-parametric test, was used for statistical analysis. A difference was determined to be significant when the p value was ≤ 0.05.

Results and discussion

Extracellular proteolytic activity in *C. neoformans* has been demonstrated in previous studies [23–26]. The present study confirmed that *C. neoformans* was able to grow on media containing protein (gelatin, casein in SMP, and BSA) as the sole nitrogen source, but only when the medium was supplemented with a carbon source. Proteinase activity was best visualised when the protein media were supplemented with a de-repressing carbon source such as fructose, xylose or galactose. In the presence of glucose, proteinase activity was not clearly visible (data not shown). This suggests that proteinase activity could be repressed in the presence of an easily assimilated carbon source.

To survey a large number of cryptococcal isolates for proteinase activity, a simple, reproducible method was developed. Previously described methods for detection of proteolytic activity on solid media included the use of an opaque substrate such as casein in SMP [35] or a 'fixative' with or without counter-staining. However, the use of an opaque substrate is relatively insensitive and does not detect low levels of protein hydrolysis in the medium. No zone of clearing was visible when SMP was used to detect protein hydrolysis by *C. neoformans* on solid media, regardless of the time of incubation, and whether SMP was present throughout the medium or only in the top layer (Table 1). This finding is consistent with the long incubation times needed in other studies to measure the proteinase activity of *C. neoformans* [25, 26].

Table 1 shows the methods used for visualising proteolytic clearing on a plate. Flooding gelatin-containing plates with trichloroacetic acid, $ZnCl_2$ or $ZnSO_4$ failed to reveal a zone of clearing. Precipitation of residual protein with trichloroacetic acid followed by counter-staining with CBB [26] was suitable for demonstrating proteolytic activity; however, the method was time-consuming and was not practicable for examining a large number of isolates (Table 1). The use of 10% $HgCl_2$ or 10% tannic acid gave the best results with a very short exposure time (Table 1). However, $HgCl_2$ has the disadvantages of toxicity to man and of killing *C. neoformans*. The use of lower concentrations (5% w/v) required longer exposure to produce an opaque complex, which then lacked the clarity achieved with the higher concentration. Isolates were no longer viable after exposure to $HgCl_2$ at either concentration. Colonies of some *C. neoformans* var. *neoformans* isolates were detached from the surface of the medium after $HgCl_2$ fixation, precluding measurement of the zone of clearing.

Table 1. Comparison of the agents used to visualise extracellular proteolysis around *C. neoformans*

Agent*	Concentration and time	Evidence of clearing†
SMP	0.5–2% for 2–14 days	None
$HgCl_2$	5% for 2 min	Satisfactory
	10% for 30 s	Good
$ZnCl_2$	5–10% for >15 min	None to poor
	15% for 10 min	Satisfactory
$ZnSO_4$	10–30% for >15 min	None to poor
TCA	20% for 2 h	None
TCA + CBB	5 h	Good
Tannic acid	5% for 2 min	Satisfactory
	10% for 15–20 s	Good

SMP, skim milk powder; TCA, trichloroacetic acid; CBB, Coomassie Brilliant Blue.

*All agents, except SMP, were tested on basal medium containing 1.0–2.0% gelatin.

†Poor, clearing evident with indistinct boundary; satisfactory, distinct boundary for some but not all isolates; good, well-defined boundary of clearing for majority of isolates.

In contrast, tannic acid is relatively non-toxic [36] and produced a precipitate comparable to that of $HgCl_2$/protein complex. Tannic acid at 10% concentration produced an opaque complex with residual protein after 15–20 s. A longer exposure to tannic acid allowed this agent to diffuse deeper into the medium and form complexes with intact protein under the colony, thereby masking evidence of proteolysis closer to the surface. This occurred even after the surface of the medium was washed thoroughly with de-ionised water. Presumably, residual tannic acid continued to complex native gelatin in the medium. Marking the position of the zone of clearing and the colony on the base of the plate, or taking a photograph of the plate immediately after exposure to tannic acid circumvented this problem.

Cells survived after a 3-min exposure to tannic acid, which was nine times longer than that required to view a zone of clearing, and colonies did not dislodge from the surface of the medium. Therefore, this would be a useful agent to screen for mutants or variants in proteinase activity (random or otherwise) in a population without the need for replica-plating.

Thirty-one isolates of *C. neoformans* var. *neoformans* and 32 of *C. neoformans* var. *gattii* were examined for extracellular proteinase activity on medium containing gelatin, and supplemented with galactose (Table 2). All except one isolate of each variety of *C. neoformans* had measurable proteinase activity, and were included in the statistical analysis of the annular size. Neither of the remaining two isolates showed clearing around or beneath the colonies. However, their ability to grow on medium containing protein as the sole nitrogen source suggests that some activity was present, possibly associated with the cryptococcal cell wall.

There did not appear to be a difference in the size of the annulus between environmental isolates and human clinical isolates of either variety of *C. neoformans*. This finding is supported by a recent report [30] which describes the proteinase activity of 40 strains of *C. neoformans* var. *neoformans* from clinical (AIDS patients) and environmental (avian guano) sources. In

Table 2. Distribution of isolates in relation to the size of the annulus produced on medium containing gelatin

Variety and source	Size of the annulus			
	no clearing*	small (⩽0.6 mm)	intermediate (0.6–1.2 mm)	large (>1.2 mm)
C. neoformans var. *gattii*				
Human clinical	1 VG-I	6 VG-I; 1 VG-II	1 VG-III	2 VG-I; 1 VG-III; 2 NT
Animal	None	4 VG-I; 1 NT	2 VG-I; 2 VG-II	None
Environmental	None	6 VG-I	1 NT	1 VG-I; 1 VG-III
Total	1	18	6	7
C. neoformans var. *neoformans*				
Human clinical	None	1 VN-Ia	1 VN-Ia; 1 NT	2 VN-Ia; 1 VN-II; 4 NT
Animal	None	None	None	14 NT
Environmental	1 NT	1 NT	2 NT	3 NT
Total	1	2	4	24

NT, not typed; VG, variety *gattii* RAPD type; VN, variety *neoformans* RAPD type.
*No clearing around or beneath the colony.

the present study, annuli around colonies of *C. neoformans* var. *neoformans* were significantly larger than those around *C. neoformans* var. *gattii*. The median annular size around isolates of *C. neoformans* var. *neoformans* was 1.6 mm, compared with 0.4 mm around isolates of *C. neoformans* var. *gattii* ($p < 0.00001$) (Fig. 1). With this as a guide, the data were divided into three categories: those with small, intermediate and large annuli (Table 2). It could be seen then that 77% (24 of 31) of *C. neoformans* var. *neoformans* isolates had an annulus >1.2 mm, while the majority of isolates (19 of 32) of *C. neoformans* var. *gattii* had an annular size <0.6 mm (Table 2). This

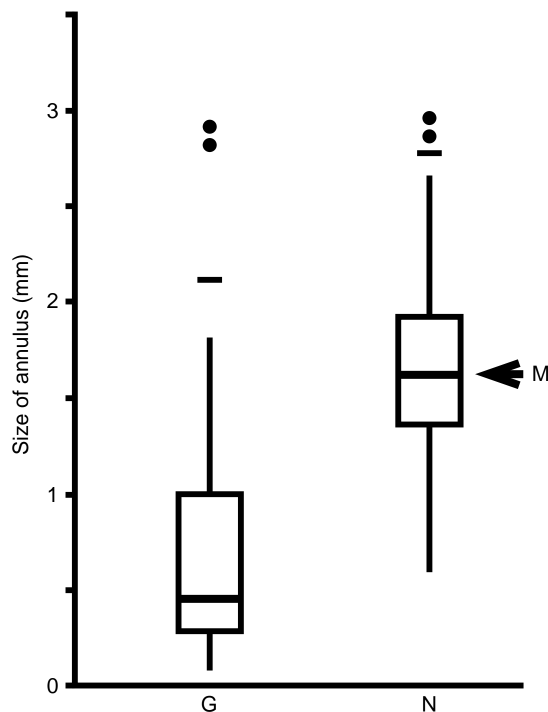

Fig. 1. Box plot of the distribution of *C. neoformans* isolates according to the size of the annulus of each isolate. The box represents the first and third quartile either side of the median, with vertical bars representing the interquartile range. G, variety *gattii* (n = 31); N, variety *neoformans* (n = 30); M, median; ●, outlier; —, boundary to interquartile range.

may reflect a difference in the way these varieties initiate infection and disseminate. *C. neoformans* var. *gattii* produces circumscribed lesions (cryptococcomas) more commonly than *C. neoformans* var. *neoformans* [2]. A deficiency in proteinase production may limit the ability of *C. neoformans* var. *gattii* to disseminate locally and into the circulation.

Within *C. neoformans* var. *gattii*, a comparison of the genetic types revealed a difference in proteinase production. The common Australian type VG-I produced a smaller annulus than the other types (VG-II and VG-III), which are mostly found outside Australia [34]. Seventeen (77%) of 22 isolates of VG-I produced annuli of ⩽0.6 mm compared with 17% (1 of 6) of VG-II and VG-III ($p < 0.02$). Few examples of the latter types were available at the time of testing, so it is not clear whether this difference in activity between the types suggests a difference in virulence or reflects a small sample size.

Differences were also found within *C. neoformans* var. *neoformans* depending on the source of isolation, with larger annuli around colonies of animal isolates (Table 2). All 14 animal isolates produced large annuli (> 1.2 mm) compared with 43% (3 of 7) of environmental isolates ($p < 0.0005$) and 70% (7 of 10) of human clinical isolates ($p < 0.01$) of *C. neoformans* var. *neoformans*. This may be related to differences in genetic types. It has been shown previously that the source of the strain may be related to genetic type, as patients with AIDS and other immunosuppressive conditions were more commonly infected with *C. neoformans* var. *neoformans* RAPD type VN-Ia [32]. An alternative explanation may be that there is a difference in the selection pressure for survival between isolates from different sources.

In summary, a rapid method to assess extracellular proteinase activity of *C. neoformans* on solid media has been developed. This method, using tannic acid, reduces the processing time from several hours [26] to a few minutes, and it is better suited for examining a

large number of isolates. Significant differences in the size of the annulus produced by each variety may relate to differences in the type, amount or diffusibility of the proteinase(s) produced, and these may influence disease development.

References

1. Franzot SP, Salkin IF, Casadevall A. *Cryptococcus neoformans* var. *grubii*: separate varietal status for *Cryptococcus neoformans* serotype A isolates. *J Clin Microbiol* 1999; **37**: 838–840.
2. Mitchell DH, Sorrell TC, Allworth AM *et al*. Cryptococcal disease of the CNS in immunocompetent hosts: influence of cryptococcal variety on clinical manifestations and outcome. *Clin Infect Dis* 1995; **20**: 611–616.
3. Speed B, Dunt D. Clinical and host differences between infections with the two varieties of *Cryptococcus neoformans*. *Clin Infect Dis* 1995; **21**: 28–34.
4. Ellis DH, Pfeiffer TJ. Natural habitat of *Cryptococcus neoformans* var. *gattii*. *J Clin Microbiol* 1990; **28**: 1642–1644.
5. Staib F, Heissenhuber M. *Cryptococcus neoformans* in bird droppings: a hygenic-epidemiological challenge. *AIDS Forschung* 1989; **4**: 649–655.
6. Ellis DH, Pfeiffer TJ. Ecology, life cycle, and infectious propagule of *Cryptococcus neoformans*. *Lancet* 1990; **336**: 923–925.
7. Sukroongreung S, Kitiniyom K, Nilakul C, Tantimavanich S. Pathogenicity of basidiospores of *Filobasidiella neoformans* var. *neoformans*. *Med Mycol* 1998; **36**: 419–424.
8. Kozel TR, Pfrommer GST, Guerlain AS, Highison BA, Highison GJ. Role of the capsule in phagocytosis of *Cryptococcus neoformans*. *Rev Infect Dis* 1988; **10** Suppl 2: S436–S439.
9. Jacobson ES, Emery HS. Catecholamine uptake, melanization, and oxygen toxicity in *Cryptococcus neoformans*. *J Bacteriol* 1991; **173**: 401–403.
10. Salas SD, Bennett JE, Kwon-Chung KJ, Perfect JR, Williamson PR. Effect of the Laccase gene, CNLAC1, on virulence of *Cryptococcus neoformans*. *J Exp Med* 1996; **184**: 377–386.
11. Odom A, Muir S, Lim E, Toffaletti DL, Perfect J, Heitman J. Calcineurin is required for virulence of *Cryptococcus neoformans*. *EMBO J* 1997; **16**: 2576–2589.
12. Chen SCA, Muller M, Zhou JZ, Wright LC, Sorrell TC. Phospholipase activity in *Cryptococcus neoformans*: a new virulence factor? *J Infect Dis* 1997; **175**: 414–420.
13. Travis J, Potempa J, Maeda H. Are bacterial proteinases pathogenic factors? *Trends Microbiol* 1995; **3**: 405–407.
14. Miyoshi S, Shinoda S. Bacterial metalloprotease as the toxic factor in infection. *J Toxicol Toxin Rev* 1997; **16**: 177–194.
15. Hube B, Monod M, Schofield DA, Brown AJP, Gow NAR. Expression of seven members of the gene family encoding secretory aspartyl proteinases in *Candida albicans*. *Mol Microbiol* 1994; **14**: 87–99.
16. Hube B, Sanglard D, Odds FC *et al*. Disruption of each of the secreted aspartyl proteinase genes SAP1, SAP2, and SAP3 of *Candida albicans* attenuates virulence. *Infect Immun* 1997; **65**: 3529–3538.
17. Sanglard D, Hube B, Monod M, Odds FC, Gow NAR. A triple deletion of the secreted aspartyl proteinase genes SAP4, SAP5, and SAP6 of *Candida albicans* causes attenuated virulence. *Infect Immun* 1997; **65**: 3539–3546.
18. Kolattukudy PE, Lee JD, Rogers LM *et al*. Evidence for possible involvement of an elastolytic serine protease in aspergillosis. *Infect Immun* 1993; **61**: 2357–2368.
19. Kothary MH, Chase T, Macmillan JD. Correlation of elastase production by some strains of *Aspergillus fumigatus* with ability to cause pulmonary invasive aspergillosis in mice. *Infect Immun* 1984; **43**: 320–325.
20. Reichard U, Monod M, Odds F, Ruchel R. Virulence of an aspergillopepsin-deficient mutant of *Aspergillus fumigatus* and evidence for another aspartic proteinase linked to the fungal cell wall. *J Med Vet Mycol* 1997; **35**: 189–196.
21. Tang CM, Cohen J, Krausz T, Van Noorden S, Holden DW. The alkaline protease of *Aspergillus fumigatus* is not a virulence determinant in two murine models of invasive pulmonary aspergillosis. *Infect Immun* 1993; **61**: 1650–1656.
22. Monod M, Paris S, Sarfati J, Jaton-Ogay K, Ave P, Latgé JP. Virulence of alkaline protease-deficient mutants of *Aspergillus fumigatus*. *FEMS Microbiol Lett* 1993; **106**: 39–46.
23. Muller HE, Sethi KK. Proteolytic activity of *Cryptococcus neoformans* against human plasma proteins. *Med Microbiol Immunol* 1972; **158**: 129–134.
24. Brueske CH. Proteolytic activity of a clinical isolate of *Cryptococcus neoformans*. *J Clin Microbiol* 1986; **23**: 631–633.
25. Chen LC, Blank ES, Casadevall A. Extracellular proteinase activity of *Cryptococcus neoformans*. *Clin Diagn Lab Immunol* 1996; **3**: 570–574.
26. Aoki S, Ito-Kuwa S, Nakamura K, Kato J, Ninomiya K, Vidotto V. Extracellular proteolytic activity of *Cryptococcus neoformans*. *Mycopathologia* 1994; **128**: 143–150.
27. Aoki K, Kajiwara S, Shinke R, Nishira H. Tannic acid staining and extraction of enzymes in polyacrylamide gel electrophoresis. *Anal Biochem* 1979; **95**: 575–578.
28. Foster AS. The use of tannic acid and iron chloride for staining cell walls in meristematic tissue. *Stain Technol* 1934; **9**: 91–92.
29. Osawa R, Walsh TP. Visual reading method for detection of bacterial tannase. *Appl Environ Microbiol* 1993; **59**: 1251–1252.
30. Leone R, Cabeli P, Sinicco A, Ito-Kuwa S, Aoki S, Vidotto V. Relationship between protease production and capsule size in *Cryptococcus neoformans*. *J Mycol Méd* 1999; **9**: 42–44.
31. Ruma P, Chen SCA, Sorrell TC, Brownlee AG. Characterization of *Cryptococcus neoformans* by random DNA amplification. *Lett Appl Microbiol* 1996; **23**: 312–316.
32. Chen SCA, Brownlee AG, Sorrell TC *et al*. Identification by random amplification of polymorphic DNA of a common molecular type of *Cryptococcus neoformans* var. *neoformans* in patients with AIDS or other immunosuppressive conditions. *J Infect Dis* 1996; **173**: 754–758.
33. Sorrell TC, Brownlee AG, Ruma P, Malik R, Pfeiffer TJ, Ellis DH. Natural environmental sources of *Cryptococcus neoformans* var. *gattii*. *J Clin Microbiol* 1996; **34**: 1261–1263.
34. Sorrell TC, Chen SCA, Ruma P *et al*. Concordance of clinical and environmental isolates of *Cryptococcus neoformans* var. *gattii* by random amplification of polymorphic DNA analysis and PCR fingerprinting. *J Clin Microbiol* 1996; **34**: 1253–1260.
35. Gornini L, Fromageot C. Une protéinase bactérienne (*Micrococcus lysodeikticus*) nécessitant l'ion calcium pour son fonctionnement. [A bacterial proteinase (from *M. lysodeikticus*) requiring calcium ion for its functioning.] *C R Hebd Séances Acad Sci* 1949; **229**: 559–561.
36. Robinson HJ, Graessle OE. Toxicity of tannic acid. *J Pharmacol Exp Ther* 1943; **77**: 63–69.