Short Communication

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High levels of hydrolytic enzymes secreted by *Candida albicans* isolates involved in respiratory infections

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Differences in production of two putative virulence factors of *Candida albicans*, phospholipase and proteinase, were determined for a large panel of clinical *C. albicans* isolates ($n = 186$) obtained from the European SENTRY programme. Seventy-two per cent of isolates produced detectable amounts of phospholipase and 95 % of isolates produced detectable amounts of proteinase. There was no clear correlation between the results of the phospholipase and proteinase assays and the geographical distribution of the isolates. However, isolates that originated from respiratory infections produced significantly higher amounts of phospholipase than isolates obtained from blood, the urinary tract or wounds/skin/soft tissue and also appeared to produce more proteinase. These virulent isolates involved in respiratory infections may originate from the oral cavity. Whether these results are caused by selection for these highly virulent isolates remains to be resolved.

Introduction

The opportunistic pathogen *Candida albicans* is considered to be the most virulent *Candida* species. Several putative virulence factors of *C. albicans* have been described, including secreted hydrolytic enzymes (Calderone & Fonzi, 2001). Two types of secreted enzyme seem to be the most important: phospholipases and secreted aspartyl proteinases (Ghannoum, 2000; De Bernardis *et al.*, 2001).

Phospholipases are most likely to contribute to the pathogenicity of *C. albicans* by damaging host-cell membranes, which aids the fungus by facilitating invasion of host tissues. Secreted aspartyl proteinases are capable of degrading epithelial and mucosal barrier proteins such as collagen, keratin and mucin, as well as antibodies, complement and cytokines. Cloning and disruption of the genes for these enzymes have shown their involvement in *Candida* virulence (Hube *et al.*, 1997; Sanglard *et al.*, 1997; Leidich *et al.*, 1998; Watts *et al.*, 1998; De Bernardis *et al.*, 1999).

Expression of virulence factors may be associated with specific characteristics of *Candida* isolates, such as geographical origin or type of infection. Knowledge of such correlations may help to understand the epidemiology of these infections, which may result in improved therapeutic regimens. Price *et al.* (1982) developed a simple egg-yolk agar plate assay for detection of phospholipase activity. Hydro-

lysis of lipid substrates present in egg-yolk results in the formation of a calcium complex with fatty acids released by the action of the secreted enzymes. The diameter of this zone of precipitation around colonies is constant for any given isolate and correlates well with a biochemical assay for the hydrolysis of phosphatidylcholine. Although this method does not detect phospholipase activity in fungal isolates that produce very low levels of phospholipase (Ghannoum, 2000), it is an excellent screening method for large numbers of isolates. We therefore used this method to investigate differences in phospholipase activity of a large collection of clinical *C. albicans* isolates from 12 European countries; the results were linked to data on geographical origin of the isolates and site of infection. For detection of proteinase activity, we incorporated BSA into yeast carbon base agar plates and measured the clearing zone after staining with Coomassie blue.

Methods

Yeast strains. *C. albicans* isolates were collected for the European SENTRY programme between 1997 and 1999. Only one isolate per patient was included. In total, 186 isolates derived from 19 medical centres in 12 European countries were studied (Table 1). One hundred and thirty-one isolates (70 %) originated from blood infections, seven (4 %) from wounds/skin/soft tissue, 25 (13 %) from the urinary tract and 23 (12 %) from respiratory infections. Most isolates were derived from the intensive care (36 %), internal medicine (15 %), surgery (14 %), paediatrics (12 %) or oncology (6 %) wards. Number of isolates derived from the most relevant hospital wards in relation to the site of infection is depicted in Table 2. Identification of isolates was performed by using CHROMagar *Candida* plates. Isolates were cultured on blood agar and subcultured on Sabouraud glucose agar (SDA) at 37 °C.

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Table 1. Geographical origin of isolates used in this study

Country	Centre	No. isolates (%)
Austria	Linz	3 (2)
France	Paris	6 (3)
	Lille	7 (4)
Germany	Freiburg	9 (5)
	Dusseldorf	7 (4)
Greece	Athens	3 (2)
Italy	Genoa	24 (13)
	Rome	17 (9)
The Netherlands	Utrecht	6 (3)
Poland	Warsaw	1 (1)
	Cracow	2 (1)
Portugal	Coimbra	23 (12)
Spain	Seville	21 (11)
	Madrid	4 (2)
	Barcelona	1 (1)
Switzerland	Lausanne	14 (8)
Turkey	Ankara	20 (11)
	Istanbul	6 (3)
United Kingdom	London	12 (6)
Total		186 (100)

Phospholipase assay. SDA plates supplemented with 1 M NaCl, 5 mM CaCl₂ and 8 % sterile egg-yolk (Oxoid) were inoculated with 1 µl sterile saline that contained approximately 10⁵ c.f.u. *C. albicans* and incubated at 37 °C for 3 days. Each isolate was tested in duplicate. Diameter of colonies and total diameter of colonies plus precipitation zones were measured. Phospholipase activity was determined by the ratio of the diameter of the colony plus precipitation zone to the diameter of the colony alone and scored as follows: —, no precipitation zone; +/-, ratio between 1.01 and 1.25; +, ratio between 1.26 and 1.50; ++, ratio between 1.51 and 1.75; +++, ratio between 1.76 and 2.00; +++++, ratio between 2.01 and 2.25.

Proteinase assay. YCB-BSA plates [1.5 % agar; 1.17 % yeast carbon base powder (Becton Dickinson); 0.2 % BSA (INstruchemie); 0.2 % glucose; 100 µl Vitox solution l⁻¹ (Oxoid)] were inoculated with 1 µl sterile saline that contained approximately 10⁵ c.f.u. *C. albicans* and incubated at 25 °C for 3 weeks. Several isolates were tested at least in duplicate. Plates were stained with 0.5 % Coomassie brilliant blue R250 (Pierce Biotechnology) in 10 % (v/v) acetic acid and 45 % (v/v) ethanol for 20 min at room temperature and destained three times with destaining solution [10 % (v/v) acetic acid; 45 % (v/v) ethanol] for 20 min at 37 °C and once with water for 20 min at 37 °C. Diameter of the colonies was measured before Coomassie staining and diameter of the

clear zones was measured after staining. Proteinase activity was determined by the ratio of the diameter of the clear zone to the diameter of the colony and scored as follows: —, no clear zone; +/-, ratio < 0.9 (clear zone smaller than colony, limited proteinase activity); +, ratio between 0.9 and 1.1 (clear zone and colony of similar size); ++, ratio > 1.1 (clear zone noticeably larger than colony).

Results and Discussion

One hundred and eighty-six isolates were tested by the phospholipase assay and 185 isolates were tested by the proteinase assay. Phospholipase activity was detected in 72 % of isolates and proteinase activity was detected in all but nine isolates (95 %). There was no clear correlation between the results of the assays and the geographical distribution of the isolates.

Duplicate testing of the isolates showed only minor differences (mean difference between duplicate tests: phospholipase assay, 0.08; proteinase assay, 0.03). This is in agreement with previous studies on the phospholipase activity of *C. albicans* isolates, in which a large variation in activity among different isolates, but a remarkably constant degree of activity of individual isolates, was reported (Price *et al.*, 1982; Samaranayake *et al.*, 1984; Kothavade & Panthaki, 1998). This activity was fairly independent of inoculum size. Our results also show a large variation in phospholipase activity among different isolates: the ratio of the diameter of the colony plus precipitation zone to that of the colony alone ranged from 1.05 to 2.36 in positive isolates.

When looking at phospholipase activity in relation to site of infection, Price *et al.* (1982) found that 55 % of blood isolates studied were positive in the assay. Furthermore, 50 % of isolates cultured from wounds and 30 % of isolates from the urinary tract were also positive. Our results show different proportions: 71 % of blood isolates, 72 % of isolates from the urinary tract and 29 % of isolates from wounds/skin/soft tissue were positive in the assay (Table 3). However, whereas Price *et al.* (1982) examined substantially more wound isolates than we did ($n = 28$ versus $n = 7$), we tested larger numbers of isolates from blood and the urinary tract (blood, $n = 131$ versus $n = 11$; urinary tract, $n = 25$ versus $n = 13$). Such differences in isolate numbers could, in part, account for the variations noted between the respective studies.

We also examined isolates that originated from respiratory infections. It appeared that this group showed the highest number of positive isolates in the phospholipase assay (87 %, 13/15).

Table 2. Number of isolates derived from different hospital wards in relation to site of infection

Source	No. isolates (%)					
	Intensive care	Internal medicine	Surgery	Paediatrics	Oncology	Other
Blood	39 (30)	22 (17)	22 (17)	18 (14)	11 (8)	19 (15)
Respiratory	18 (78)	0 (0)	0 (0)	0 (0)	0 (0)	5 (22)
Urinary tract	8 (32)	4 (16)	3 (12)	4 (16)	0 (0)	6 (24)
Wound/skin/soft tissue	2 (29)	1 (14)	2 (29)	0 (0)	0 (0)	2 (29)
						7 (100)

Table 3. Results of phospholipase and proteinase assays in relation to site of infection

Assay (site of infection)	No. isolates (%)						Total
	–	+/–	+	++	+++	++++	
Phospholipase:							
Blood	38 (29)	10 (8)	36 (27)	22 (17)	19 (15)	6 (5)	131 (100)
Respiratory	3 (13)	1 (4)	5 (22)	8 (35)	5 (22)	1 (4)	23 (100)
Urinary tract	7 (28)	2 (8)	9 (36)	3 (12)	3 (12)	1 (4)	25 (100)
Wound/skin/soft tissue	5 (71)	0 (0)	1 (14)	0 (0)	1 (14)	0 (0)	7 (100)
Proteinase:							
Blood	5 (4)	21 (16)	83 (63)	22 (17)	–	–	131 (100)
Respiratory	0 (0)	1 (4)	14 (61)	8 (35)	–	–	23 (100)
Urinary tract	2 (8)	3 (13)	17 (71)	2 (8)	–	–	24 (100)
Wound/skin/soft tissue	2 (29)	0 (0)	4 (57)	1 (14)	–	–	7 (100)

$n = 23$). Furthermore, 61 % of these isolates were among the higher producers (++, +++ or ++++). In comparison, of all strains obtained from blood ($n = 131$), the urinary tract ($n = 25$) or wounds/skin/soft tissue ($n = 7$) that were tested in the phospholipase assay, most isolates either were negative or produced only low amounts of phospholipase (–, +/- or +; blood, 64 %; urinary tract, 72 %; wounds/skin/soft tissue, 85 %) (Table 3). This difference was statistically significant [$P = 0.042$; Pearson χ^2 test (exact)]. Although not statistically significant, a similar trend was observed for the proteinase assay: all isolates obtained from respiratory infections were positive in the proteinase assay and 96 % of these isolates produced considerable amounts of proteinase (+ or ++). For isolates obtained from the other sources (blood, urinary tract or wounds/skin/soft tissue), this proportion was 80, 79 and 73 %, respectively (Table 3). According to fingerprinting data obtained with amplified fragment length polymorphism (AFLP) analysis, only two isolates that originated from respiratory infections (from Genoa, Italy) were identical. The patterns of all other respiratory infection isolates showed clear differences. Similarly, patterns of isolates from other sources showed no sign of bias due to hospital outbreaks (results not shown).

Our results are supported by those of Kantarcioglu & Yucel (2002). Although the focus of their study was on the differences in phospholipase and proteinase production between different *Candida* species, it can be concluded from their data that *Candida* isolates that originated from the respiratory tract showed the highest mean production of both phospholipase and proteinase. Furthermore, compared to other sources (oral cavity, urogenital system and blood), this site of infection showed the highest number of positive isolates in the phospholipase assay. Price *et al.* (1982) found that blood isolates were the highest producers of phospholipase; however, their study did not include isolates from the respiratory tract.

It is possible that our findings are related to those of earlier reports by Samaranayake *et al.* (1984) and Kothavade & Panthaki (1998), which mention relatively high numbers of

phospholipase producers among clinical oral *C. albicans* isolates (79 and 89 %, respectively). An exceptionally high proportion (78 %) of our respiratory infection isolates were derived from patients in intensive care. For the other three sources, this proportion was approximately 30 % (Table 2). Although data are lacking, it seems legitimate to assume that many of these patients were ventilated mechanically. In that case, *C. albicans* isolates that cause respiratory infections may very well originate from the patient's own oral cavity.

It is interesting to note that, whereas oral *C. albicans* isolates from healthy volunteers show relatively low phospholipase activity, clinical isolates from oral cavities of patients suffering from oral candidiasis produce relatively high amounts of this enzyme (Samaranayake *et al.*, 1984; Ibrahim *et al.*, 1995; Kothavade & Panthaki, 1998). Although Kantarcioglu & Yucel (2002) report relatively low phospholipase and proteinase production among oral *Candida* isolates, their isolates were obtained from patients suspected of invasive fungal infection, as opposed to patients suffering from oral candidiasis. These oral isolates may, therefore, not be very different from isolates from healthy individuals. Furthermore, oral *C. albicans* isolates from human immunodeficiency virus (HIV)-positive individuals are known to cause unusually severe infections. These isolates also produce extremely high amounts of proteinase (Ollert *et al.*, 1995; De Bernardis *et al.*, 1996). It is hypothesized that these infections are attributable to the selection of commensal *C. albicans* isolates that are characterized by higher virulence. It is a tempting idea that these more virulent isolates also have increased potential to cause respiratory infections in intensive-care patients. Underlying mechanisms behind the selection of these highly virulent strains have not yet been determined.

Proteinase production by *C. albicans* depends not only on strain type or type of infection, but also on phenotypic switch type, environmental conditions and even the stage of infection (De Bernardis *et al.*, 2001). Therefore, caution must be employed in interpretation of proteinase assays. Although the chosen assays were crude, in particular the

proteinase method, it is noteworthy that the results of both assays indicate higher virulence for isolates involved in respiratory infections. Whether this is caused by selection of more virulent isolates that are part of the patients' commensal flora remains to be resolved.

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