Vaginopathic and proteolytic *Candida* species in outpatients attending a gynaecology clinic

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Abstract

Non-pregnant, non-diabetic outpatients were examined for the presence of pathogenic vaginal yeasts to determine if a correlation existed between a specific yeast and clinical disease. Yeasts were isolated as single vaginal species from 186 of 228 subjects with clinically diagnosed candidal vaginitis, as well as from 122 out of 380 asymptomatic, age-matched controls. Apart from *Candida albicans* and *C glabrata*, other prevalent species were *C krusei*, *C parapsilosis* and *Saccharomyces cerevisiae* which accounted for 9.2%, 6.0% and 5.4%, and 9.0%, 2.4% and 19.7%, of yeasts from patients and carriers, respectively. Only *C albicans* and *C parapsilosis* were significantly more common in those with vaginitis. Only the isolates of these two species secreted aspartyl proteinase in vitro, and the amount of the enzymes secreted by the isolates from patients was significantly higher than that secreted by the isolates from carriers. These two species consistently produced vaginal infection in pseudoestrus rats, whereas none of the non-proteolytic species tested (*C glabrata*, *C krusei*, and *S cerevisiae*) colonised the vagina in these rats.

Proteinase secretion correlated with experimental vaginal infection; it could also be a reliable factor for distinguishing clinically active infection from asymptomatic fungal carriage.

Candidal vaginitis continues to pose several aetiological, diagnostic, and pathogenetic challenges.¹⁻³ While many authors have estimated the incidence of candidal vaginitis and recorded the species of *Candida* more often among patients with vaginitis, reports based on careful clinical and mycological observations are relatively few. *C albicans* and *C glabrata* are believed to be the only or the most common agents of candidal vaginitis, although many other species of this fungus have been isolated from the vagina of infected subjects.¹ In particular, *C parapsilosis* has recently been shown to have considerable pathogenic potential in experimentally induced infection in pseudoestrus rats whose ovaries had been removed.^{4,5}

No agreement has been reached as to whether the presence of even the most pathogenic species (*C albicans*) in the vagina is always associated with vaginitis, or whether

carriage of *Candida* sp is normal but harmless. For some authors *C albicans* is rarely found in the vagina in the absence of local pathology.¹ McCormack *et al* observed symptoms in 67% of women whose vaginal cultures contained yeasts and they concluded that vaginal colonisation by yeasts is commonly associated with vulvovaginal symptoms.⁶ On the other hand, other authors have convincingly shown that the prevalence of vaginal yeasts was similar in women with and without vaginal symptoms.^{7,8} Odds¹ and Sobel^{3,9} have recently reviewed the evidence for normal vaginal carriage of *Candida* sp.

The pathogenesis of candidal vaginitis is also unclear. Although several risk factors have been identified, such as pregnancy and diabetes, and treatment with corticosteroids and broad spectrum antibiotics,^{1,9} little is known about the virulence factors of the yeast responsible for vaginal colonisation or vaginitis, and the mechanisms which determine the transition from asymptomatic yeast carriage to vaginitis.

Methods

Non-pregnant, non-diabetic women (n = 608), aged 19 to 63 years and attending the Center for Tumor Prevention in Sant'Andrea Hospital, Rome, were enrolled in this study. The criteria for inclusion were as follows. The first five women who presented to the Center each day between 1986 and 89, asking for a gynaecological examination for any reason, except those who were: (i) younger than 18 and 65 years or older; (ii) pregnant; (iii) diabetic; (iv) taking antibiotics, or topical antifungal agents two months before examination; (v) taking vaginal contraceptives the week preceding the visit to the Center. Each subject completed a questionnaire on demographic, social, sexual and medical data. Around 10% of women regularly used contraception, a percentage that was similar among patients and controls.

All subjects with non-candidal vaginitis (based on the criteria reported by Sweet¹⁰), as well as those from whose vagina more than a single yeast species was isolated, were excluded. The differential diagnosis of candidal vaginitis was based on the isolation of a yeast species from subjects with a typical vaginal discharge (clumpy, cottage-cheese appearance) and intense vulvovaginal pruritis, accompanied by vulvar erythema and dyspareunia. All other yeast positive (by culture) asymptomatic subjects served as controls. All eligible subjects gave their written, informed consent.

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YEAST ISOLATION AND IDENTIFICATION

Two high vaginal samples were taken with cotton tipped swabs from all patients. One swab was immediately smeared on to a glass slide, fixed, and stained with periodic acid-Schiff (PAS) for microscopical examination. The other was kept in sterile physiological saline and transferred to the laboratory where it was seeded on plates of Sabouraud-dextrose agar (BBL, Baltimore, Maryland) with added chloramphenicol (50 µg/ml) and Mycosel agar and then immersed into enrichment broth. All cultures were incubated for 48–72 hours at 30°C. All isolates were preliminarily identified on the basis of their assimilation or fermentation profiles in the API 20C system (BioMérieux), capacity to form germ-tubes in serum, morphology and chlamydospores in corn-meal agar. The identification was confirmed by an expanded battery of assimilation-fermentation tests according to Meyer *et al.*¹¹ Serological analysis according to Tsuchiya *et al.*¹² was also performed as an aid to the final identification and serotyping of *C. albicans*.

PROTEINASE SECRETION IN VITRO

All isolates were tested for their ability to grow and produce clear zones of hydrolysis in bovine serum albumin (BSA) agar using previously established methods.¹³ Acid proteinase activity was scored as follows: – or ± when no visible or a very limited clarification of the agar around the colony was present; 1+ when visible proteolysis was observed (1–2 mm); and 2+ when agar clarification largely exceeded (3–5 mm) the margin of the colony. Several isolates of *C. albicans* and all isolates of *C. parapsilosis* were also tested for proteinase assay in liquid medium containing bovine haemoglobin, as described previously.^{13,14}

EXPERIMENTAL VAGINITIS IN RATS

For experimentally induced vaginal infections, female Wistar rats, weighing 80–100 g, whose ovaries had been removed, were obtained from Charles River Breeding Laboratories and injected subcutaneously with 0.5 mg of oestradiol benzoate (Benzatrone; Samil, Rome, Italy) every two days. Six days after the first dose of oestradiol the animals were inoculated intravaginally with 10⁷ cells (0.1 ml volume) of each yeast isolate tested. The yeast had been grown to stationary phase in YEPD medium at 28°C.⁵ It was then harvested by low speed centrifugation, washed, and suspended to the required number in phosphate buffered saline. Yeast cells were injected into the vaginal cavity through a syringe equipped with a multi-purpose calibrated tip (Combitip; PBI, Milan, Italy). Vaginal fluid was taken from each animal every two days, with a special purpose calibrated (1 µl) plastic loop (Dispoinoc; PBI). Some aliquots were used for microscopical examination; other specimens (one vaginal sample per rat per culture) were used for quantitation of vaginal colonisation. For this, the contents of each loop were vigorously shaken in 0.1 ml of phosphate buffered saline and the portions streaked over plates containing Sabouraud glucose agar plus chloram-

phenicol and incubated for 48–72 hours at 28°C. At intervals during vaginal infection, colonies developed on the mycological medium were subjected to confirmatory species identification according to the diagnostic criteria reported above.

Differences in the frequency with which each yeast species was isolated from the different groups were assessed for significance using the χ^2 method. Differences in proteinase secretion in liquid medium were assessed by Student's *t* test; whereas the differences in proteinase secretion in BSA agar were assessed by the χ^2 test or Fisher's exact probability methods, as appropriate. Differences in yeast cell counts (CFU) in infected vaginal fluids were assessed by Student's *t* test.

Results

PREVALENCE AND DISTRIBUTION OF VAGINAL YEAST SPECIES

Yeasts were isolated as single vaginal species from 186 of 228 patients with clinical symptoms and signs of candidal vaginitis. Either two or more yeasts (generally *C. albicans* plus *C. krusei*, or *C. parapsilosis*; *n* = 10 subjects) or no yeast (*n* = 32 subjects) were isolated from the remaining 42 patients. Yeasts were also isolated as single vaginal species from 122 of 380 asymptomatic subjects, the remaining 258 being negative for vaginal yeast isolation. Table 1 shows the distribution of species and their relative proportions in the two groups of subjects.

In the group with vaginitis *C. albicans* was the predominant species with a prevalence of almost 50%. As expected, the second most frequently isolated species was *C. glabrata* (slightly more than 20%), followed by, in descending rank of prevalence, *C. krusei*, *C. parapsilosis*, *C. kefyr* and *C. tropicalis*. *Saccharomyces cerevisiae* and a miscellanea of other non-pathogenic *Candida* species represented only 7.5% of all isolates. Among asymptomatic, control subjects *C. albicans* and *C. glabrata* were isolated with a similar frequency (around 20%), which was also similar to the percentage isolation of *S. cerevisiae* or miscellaneous yeast species. Overall, the seven aforementioned yeast species accounted for more than 92% of all isolations.

Table 1 also shows that *C. albicans* and *C. parapsilosis* were isolated significantly more frequently from those with vaginitis than from carriers; the reverse was true for *S. cerevisiae* and the miscellaneous yeasts: in particular, *Rhodotorula* spp were isolated only from asymptomatic subjects. There was no significant difference between the two groups of subjects examined in terms of the isolation prevalence of all other species, including *C. glabrata* (table 1).

IN VITRO PROTEINASE SECRETION

The ability to secrete an acid (aspartyl) proteinase, a potential virulence factor in the pathogenesis of candidal vaginitis,^{13,14} was assessed in vitro using both BSA agar (BSA-P) and haemoglobin bovine liquid medium (HB-P).¹⁴

Table 1 Correlation between clinical vaginitis, vaginal carriage, and yeast isolation

Yeasts isolated	Women with signs and symptoms of candidal vaginitis		Asymptomatic women (carriers)		p value
	N=	% Isolates	N=	% Isolates	
<i>Candida albicans</i>	92	49.9	24	19.7	<0.01
<i>Candida glabrata</i>	41	22.3	26	21.3	NS
<i>Candida krusei</i>	17	9.2	11	9.0	NS
<i>Candida parapsilosis</i>	13	6.0	3	2.4	<0.05
<i>Candida kefyr</i>	5	2.7	7	5.7	NS
<i>Candida tropicalis</i>	4	2.1	5	4.1	NS
<i>Saccharomyces cerevisiae</i>	10	5.4	24	19.7	<0.05
Others	4*	2.1	22†	18.0	<0.01
Total	186		122		

**C. famata* 1, 3 unidentified.†*Rhodotorula* sp. 13, *C. famata* 2, *C. humicola* 1, *C. lipolytica* 1, *C. lambica* 1, *Trichosporon beigeli* 1, *Aureobasidium* sp. 1.

All or most of the isolates of each species were tested by at least one of the above methods. Table 2 shows that all tested isolates of *C. albicans* and *C. parapsilosis* secreted a measurable amount of acid proteinase (one isolate of *C. parapsilosis* was negative on BSA agar but positive on HB liquid medium). Most of the few vaginal isolates of *C. tropicalis* and *C. kefyr*, and all the isolates of *C. glabrata* and *C. krusei* did not secrete the enzyme in vitro. Table 2 also shows that both BSA-P and HB-P of *C. albicans* and *C. parapsilosis*, were significantly higher in the isolates from those with vaginitis than in those from carriers; no significant difference was noticed between the proteinase activity of *C. albicans* and that of *C. parapsilosis*, regardless of their isolation source.

EXPERIMENTALLY INDUCED RAT VAGINITIS

While the potential of both *C. albicans* and *C. parapsilosis* to produce vaginal infection in rats

Experimentally induced rat vaginitis with vaginal isolates of various *Candida* species. Groups of five rats were infected (day 0) with 10^7 cells of *Candida albicans* (isolate SA-40; ●—●), *C. parapsilosis* (SA-19; △—△), *C. glabrata* (SA-380; ■—■), *C. krusei* (SA-301; ○—○), and *S. cerevisiae* (SA-264; □—□). *Candida* numbers are expressed as mean CFU (SE) (bar)/ml of vaginal fluid of infected ($\geq 10^3$ CFU/ml) rats.

without ovaries and maintained with oestradiol is already established,⁵¹⁴ no or very few data exist on the experimental potential to cause vaginitis of *C. glabrata*, *C. krusei*, or *S. cerevisiae*. Because of the high prevalence of these three

Table 2 Acid proteinase activity¹³ in vitro by vaginal isolates of *Candida* spp

Species	No of isolates tested	BSA-P of isolates from:						HB-P (Δ OD/ml) of isolates from:		t test
		Patients			Controls			Patients (mean (SD))	Controls (mean (SD))	
		2+	1+	0	2+	1+	0			
<i>C. albicans</i>	112	44	34**	0	3	31**	0	1.06 (0.24)*	0.78 (0.12)†	p < 0.001
<i>C. parapsilosis</i>	17	9	1††	1	1	5††	0	1.14 (0.33)‡	0.80 (0.13)††	p < 0.01
<i>C. glabrata</i>	78	0	0	41	0	0	37			
<i>C. krusei</i>	34	0	0	17	0	0	17			
<i>C. tropicalis</i>	9	0	3	1	0	1	4			
<i>C. kefyr</i>	11	0	2	3	0	1	5			

*50 different isolates, 70 independent determinations.

†34 isolates, 38 independent determinations.

‡11 isolates, 12 determinations.

††3 isolates, 8 determinations.

**p < 0.01, χ^2 test, comparing the ratios 2+ : 1+.

††p < 0.05, Fisher's exact test, comparing the ratios 2+ : 1+.

Table 3 Quantitation of vaginal colonisation (CFU (SD) $\times 10^{-3}$ yeast cells/ml vaginal fluid) in pseudoestrus rats infected with *Candida* isolates

Fungal isolate	Days after challenge				
	1	2	7	14	21
* <i>C. albicans</i>	>100 (5/5)†	>100 (5/5)	80 (2.5) (5/5)	40 (3.8) (5/5)	26 (2.5) (3/5)
* <i>C. parapsilosis</i>	>100 (5/5)	>100 (5/5)		23.7 (2.1) (2/5)	19 (3.7) (2/5)
<i>C. glabrata</i>	>100 (5/5)	15.8 (4.0) (5/5)	<1 (0/5)	<1 (0/5)	<1 (0/5)
<i>C. glabrata</i>	57.2 (17.9) (5/5)	16.0 (1.8) (4/5)	<1 (0/5)	<1 (0/5)	<1 (0/5)
* <i>C. glabrata</i>	>100 (5/5)	55.4 (19) (5/5)	<1 (0/5)		
<i>C. glabrata</i>	>100 (5/5)	20.0 (4.0) (4/5)			
* <i>C. krusei</i>	>100 (5/5)	34 (16.0) (4/5)	1 (1/5)	<1 (0/5)	<1 (0/5)
<i>C. krusei</i>	29 (17.2) (4/5)	24.2 (22) (3/5)	<1 (0/5)		
<i>C. krusei</i>	>100 (5/5)	10.6 (5.6) (5/5)	<1 (0/5)	<1 (0/5)	<1 (0/5)
* <i>S. cerevisiae</i>	76 (20.8) (4/5)		<1 (0/5)		
<i>C. krusei</i>	58 (13.2) (5/5)	38.5 (21.9) (4/5)	2 (1/5)	<1 (0/5)	<1 (0/5)

* Shown in kinetics, in an independent experiment, in the figure.

†Rats infected/total.

yeasts in our subjects, we tested randomly selected isolates of these three species in a previously established rat vaginitis model, and compared them with two isolates of *C. albicans* and *C. parapsilosis* as positive controls.

Table 3 summarises the individual data obtained for each isolate, inclusive of both the total cfu counts and the number of rats infected ($> 10^3$ cells/ml of vaginal fluid) over total numbers, over three weeks, after vaginal challenge of 10^7 cells. The figure outlines the kinetics of infection with representative isolates of each species. Altogether, the data show clearly that the isolates of *C. krusei* and *C. glabrata*, like those of *S. cerevisiae*, were eliminated from the vagina of infected rats within the first week after intravaginal challenge. On the contrary, both *C. parapsilosis* and *C. albicans* persisted to infective concentrations ($> 10^3$ cfu/ml), significantly longer, confirming previous results. No significant differences were found among the various isolates of *C. glabrata* or *C. krusei* in the rate of elimination of yeast cells from the vagina, measured both as total cfu count and as a proportion of infected rats.

Discussion

One hundred and eighty six cases of clinically and mycologically defined candidal vaginitis were matched with 122 asymptomatic carriers among subjects attending a gynaecological Center in Rome. All the women were not pregnant and non-diabetic, and no difference between the two groups of subjects was present in the use of method of birth control. Thus the results may offer a reasonable indication of the prevalence of defined yeast species in vaginitis, as opposed to vaginal carriage of the same species, in the population examined. Despite a large number of studies,¹ very few attempts have been made to evaluate prevalence and role of vaginal isolates other than *C. albicans* as pathogenic agents, and to assess their virulence and experimental pathogenicity in animals.

A remarkable aspect of the population we examined was that about half the yeasts isolated from typical cases of candidal vaginitis were not *C. albicans*, and some were non-*Candida* (*Saccharomyces*) species. The increased proportion of these apparently uncommon, vaginal species allowed us to evaluate preferential association of these species with carriage or overt disease. Interestingly, a well known aetiological agent of candidal vaginitis, *C. glabrata*, had a high comparable prevalence in both patients and carriers, whereas only *C. parapsilosis* resembled *C. albicans* in being significantly more frequently isolated from patients than carriers. This strongly suggests a correlation between *C. parapsilosis* and candidal vaginitis, in keeping with our previous results obtained in oophorectomised, pseudoestrus rats.⁴⁵ This also suggests that both *C. albicans* and *C. parapsilosis* may have special virulence characteristics which facilitate the transition from harmless carriage to pathogenicity. One possible virulence factor which seems to belong to these two species and to differentiate them from all other prevalent vaginal species is the secretion

of an aspartyl proteinase, an enzyme capable of hydrolysing mucosal IgA, the most effective barrier against infection in the vagina.¹⁵ In this survey we extended previous observations^{5,13} to show that all vaginal isolates of *C. albicans* and *C. parapsilosis* produce a remarkable amount of proteinase in vitro. Importantly, acid proteinase secretion by the isolates from the patients with vaginitis was significantly higher than that by the isolates from controls, confirming preliminary data on *C. albicans* in a limited sample of subjects.¹³ The data obtained from the experimental infections of rats whose ovaries had been removed also support the idea that proteolysis may be particularly important in vaginitis. In fact, only the two proteolytic species of *Candida* (*C. albicans* and *C. parapsilosis*) caused an extended and prolonged vaginal colonisation in these rats. As these two species differ in many other potential virulence factors,¹⁶ their ability to give a rat vaginal infection, comparable in extent and duration, is rather surprising, and may indicate that in experimental rat vaginitis proteinase secretion is a dominant virulence determinant. This seems at variance with human vaginitis where the concerted action of a number of virulence factors seems more likely.^{9,17}

It has been established that vaginitis in rats depends on induction (by pseudoestrus) of keratinisation of vaginal epithelia,^{17,18} and it is well known that the aspartyl proteinase produced by *Candida* sp is potently keratinolytic.¹⁹ This may also explain why none of the non-proteinase producer yeasts, including some which are clearly implicated in human vaginitis, was able to induce vaginal infection in rats. Altogether, the data of this study lead us to speculate that women harbouring acid proteinase producing species of *Candida* (*C. albicans* and *C. parapsilosis*) might be at an increased risk of developing clinically active vaginitis.

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Eponyms in pathology

SCHWANN, Theodor (1810-1882) was a German physiologist, born near Dusseldorf. He studied medicine in Bonn, graduating in 1834. He then worked in Wurzburg and Berlin, where for five years he was a pupil of Johannes Muller (of Mullerian duct fame). It was there that he did his best work, and in 1837 published his classic declaration of cell theory in a book on the similarities of growth and structure between plants and animals. In 1839 he described the large nucleated cells of the nerve sheath of peripheral nerve fibres (Schwann cells) which are now known to produce myelin.

In the same year he was appointed Professor of physiology at Louvain, Belgium, moving to Liège in 1849 as Professor of comparative anatomy before his retirement in 1880. Among his other achievements, he discovered pepsin (1835), showed that bile is necessary for digestion, and was the first to show that fermentation involved living organisms (being acknowledged by Pasteur for this). Soon after leaving Berlin, however, he abandoned rationalism and became a mystic, doing very little original work thereafter.