A Simplified Technique for Evaluating the Adherence of Yeasts to Human Vaginal Epithelial Cells

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Thousands of women all over the world annually suffer of vulvovaginal candidiasis (VVC), an infection caused by yeasts, which mainly affect the mucosa of the vulva and vagina. The adherence of the yeasts to the mucosae is an essential step for colonization and predisposes the start of the infectious process. In this study, a technique capable of evaluating the adhesion of yeasts to human vaginal epithelial cells (HVEC) was employed. Twenty-five vaginal yeast isolates (10 *Candida albicans*, nine *C. glabrata*, two *C. parapsilosis*, one *C. tropicalis*, two *Saccharomyces cerevisiae*, and one *Trichosporon* sp.) were evaluated. A suspension of each yeast was

co-incubated with HVEC obtained from a healthy donor in the ovulatory phase. After 1 hr, smears were made, stained with crystal violet and Papanicolaou, and the number of yeasts that adhered to 600 HVEC was evaluated. The adhesion of *C. albicans* was significantly greater than that of the other species and occurred mainly in the intermediate HVEC, rather than the superficial. In addition, the proposed technique, easy to execute and of low cost showed to be reproducible and enables the determination of the adherence capacity of different isolates, whose adhesion was confirmed by scanning electron microscopy. J. Clin. Lab. Anal. 20:195–203, 2006. © 2006 Wiley-Liss, Inc.

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INTRODUCTION

Vulvovaginal candidiasis (VVC) is an infectious process caused by the abnormal growth of yeasts in the mucosa of the female genital tract. It is an infection of the vulva and vagina caused by yeasts that normally inhabit the vaginal mucosa, but which may become pathogenic in predisposing conditions (1). *Candida albicans* has been considered the main agent, responsible for 80–90% of VVC (2,3). However, more recent data show the increase of other species, called non-*C. albicans*, being responsible for 40% of VVC (4).

According to Mardh et al. (5), approximately three-quarters of all women will experience an episode of vulvovaginal candidosis at least once in their life, and 5–10% of them will have more than one attack.

In addition to factors relating to the host, there are intrinsic properties of the yeasts that promote their

ability to cause VVC. Adherence to host tissues and production of extracellular enzymes are two of the more important virulent attributes of the *Candida* species. However, the specific mechanisms by which the yeasts cause VVC are not completely understood (6,7).

The adherence of the yeasts to the vaginal mucosa is recognized as an essential step in microbial colonization and a key event in the start of the pathogenic process, as prior colonization is accepted as a predisposing factor to

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the infectious process (8). In support of this theory, non-adherent *C. albicans* isolates cause less disease in animal models (9). The adherence mechanisms of these yeasts to human tissues are varied and occur as the result of the *Candida*-host cellular recognition systems, which are extremely complex and involve a variety of factors (10).

Alterations in the monthly cycles of ovarian hormones, estrogen, and progesterone of women of reproductive age reflect the maturation of the vaginal epithelium (non-keratinized stratified squamous). In the first phase of the menstrual cycle (proliferative phase), a progressively increasing secretion of estrogen occurs until ovulation. This functions as a type of growth hormone for the epithelium and leads to its maturation, reflected in the predomination of superficial epithelial cells (SC). After ovulation (luteal phase), progesterone is secreted in a greater quantity than estrogen and, in physiological conditions, this hormone has an anti-estrogen effect on the epithelium, interrupting its maturation and causing the appearance of intermediate epithelial cells (IC). This way, the cyclical hormonal changes of these two hormones reflect the change in the ratio of SC to IC (11). There are clinical observations that VVC occurs more frequently during the luteal phase of the menstrual cycle (12). Some studies on the adherence capacity of yeasts to IC and SC have been carried out but their results were not conclusive (13,14).

The technique of staining with Papanicolaou (Pap) is extensively used in cervical cytology and is based in the affinity of acid dyes (light green, eosin, and orange G) to the basic cytoplasmic constituents, for example keratin. Therefore, this method of staining enables the differentiation of human vaginal epithelial cells (HVEC) by their morphology and staining. IC are polygonal with clear outlines and a well-defined, round or oval vesicular nucleus. Their cytoplasm is light blue, green, or violet in color. SC are polygonal, with transparent cytoplasm and a pycnotic nucleus. The cytoplasm is often pink stained, although some may show a pale blue color (11,15).

The objective of this study was to standardize a technique, based on Pap stain, for evaluating the adherence of yeasts to HVEC and validate this methodology using 25 vaginal isolates.

MATERIALS AND METHODS

Collection and Preparation of Epithelial Cells

The HVEC were obtained from a healthy donor of reproductive age with a regular menstrual cycle, and always on the 14th day of the cycle (ovulatory phase). The donor was not using douches, vaginal medicines, or hormonal contraceptives. The HVEC were collected

using a vaginal speculum (Vagispec, Jaragua do Sul, Brazil) by scraping the side wall of the vagina with a sterilized Ayre spatula. The material obtained was re-suspended in 10 mL phosphate buffer saline 0.01 M, pH 7.2 (PBS), centrifuged at 1,500 rpm for 5 min, and washed three times with the same volume of PBS. The sediment was examined by common optical microscopy to ensure there was no colonization by yeasts. A suspension containing 2×10^5 HVEC/mL was prepared using a Neubauer hemocytometer chamber (Boeco, Hamburg, Germany), according to Bailey et al. (16) and Williams et al. (17). The adherence assays were started up to 30 min after the cell collections.

Yeasts

Twenty-five yeasts isolated from vaginal exudate were used: 10 *Candida albicans*, nine *C. glabrata*, two *C. parapsilosis*, one *C. tropicalis*, two *Saccharomyces cerevisiae*, and one *Trichosporon* sp. The samples were taken from the yeast bank of the Medical Mycology Section of the Laboratory of Teaching and Research in Clinical Analysis of the State University of Maringá, Paraná, Brazil, and kept in distilled water under refrigeration at 4°C. The yeasts had been previously identified by the classic method (18) and confirmed using rDNA sequences according to Sugita et al. (19).

STANDARDIZATION OF THE YEAST SUSPENSIONS

The yeast inoculum of two *C. albicans* isolates was prepared from the stock culture plated onto Sabouraud Dextrose Agar (Difco, Detroit, MI) and incubated for 48 hr at 25°C. One loop full of the culture was inoculated in yeast nitrogen base (YNB broth; Difco), supplemented with 50 mM of galactose, and it was incubated for 24 hr at 37°C. After incubation, the fungal growth was centrifuged at 1,500 rpm for 5 min, the supernatant discarded, and the sediment washed three times with 5 mL of PBS. A serial dilution from 5×10^3 to 5×10^7 yeasts/mL was prepared in PBS and they were counted using the Neubauer hemocytometer chamber.

Adherence Assay

The technique recommended by Kennedy (20) was used as a basis for the adherence assay, substituting filtration through polycarbonate membrane filters (Millipore) by centrifugation at 1,500 rpm for 5 min, according to the modified urocytogram technique (21). Equal volumes of HVEC and fungal cell suspensions (0.5 mL) in different dilutions were co-incubated in conical glass tubes for 1 hr at 37°C under agitation at 70 rpm in a shaker (NT 712; Nova Técnica, Piracicaba, Brazil). The

preparations were centrifuged at 1,500 rpm for 5 min. The sediments were washed five times in 3 mL of PBS to remove non-adhered yeasts. After the last wash, the excess supernatant was discarded and 50 μ L of the sediment was carefully spread on 26 $\times$ 76 mm microscope slides and left to dry at room temperature. The smears were stained with crystal violet (Merck, Darmstadt, Germany) for 1 min, washed with water, and immediately stained using the Papanicolaou method (Merck) according to Bibbo (15). One microscope slide per experiment was stained with Pap but without crystal violet. The microscope slides were covered with 24 $\times$ 60 mm coverslips, using synthetic balsam Entellan to obtain permanent smears. All the procedures were repeated in triplicate on three separate occasions. The images were obtained through software Cytoviewer for Cytopathology (Labho, São Paulo), a system for the analysis of images and diagnoses.

Standardization of the Microscopic Criteria of Adhesion

The smears were examined by common optical microscopy, amplification $\times$ 400, to determine the concentration of the yeasts, which provided a better visualization of adherence to the HVEC for later use in further assays. For each experiment, the number of yeasts adhered to 400 HVEC per microscope slide (200 SC and 200 IC) were counted in consecutive microscopic fields, giving a total number of yeasts adhered to 1,200 cells per experiment (600 CS and 600 IC). Isolated or single budding yeasts were considered as fungal units; pseudohyphae and aggregations of yeasts were excluded from the count. The HVEC were differentiated into superficial or intermediate cells by the Pap method, according to Takahashi (11) and Bibbo (15). The evaluation was carried out in microscopic fields that did not show overlaid HVEC or stain artifacts. Only yeasts that were in direct contact with the HVEC were considered as adhered yeasts.

Scanning Electron Microscopy (SEM)

The adherence assay was carried out with an isolate of *C. albicans*; the resultant sediment was fixed in glutaraldehyde 2.5% in 0.1 M cacodylate buffer. The material was centrifuged and dehydrated in increasing grades of alcoholic solution, and the critical point was reached in a Balzers CPD-010 (Balzers Instruments, Balzers, Liechtenstein) with carbonic gas. Then gold metallization was carried out in a Balzers SCD-030 (Balzers Instruments, Balzers, Liechtenstein). The analyses and documentation were carried out in an electronic microscope JEOL-JSM 6360 LV Jeol Ltd., Tokyo, Japan in the Centre of Electronic Microscopy–Federal University of Paraná, Paraná, Brazil.

Statistical Analysis

An analysis of variance (ANOVA) and Scheffe test were used for multiple comparison using the computer software Statistica, version 6.0 (Stat Soft, Inc. 2001, Tulsa, OK). *P*-values <0.05 were considered significant.

RESULTS

The methodology proposed was efficient at demonstrating the in vitro adherence of vaginal yeast isolates to HVEC (Fig. 1). The inclusion of crystal violet verified to Pap technique a greater capacity to show the yeasts without interfering in the staining of the epithelial cells. Fig. 1d–f shows the yeasts in direct contact with the HVEC, evidenced by the re-entrances at the point of adhesion.

In the scanning electron microscopy, epithelial cells in a good state of preservation were observed with defined outlines, well-demarcated plasma membranes, typical epithelial cell morphology, and various associated yeasts (Fig. 2a). After 1 hr of interaction, areas of contact appeared as regions of continuity, with interdigitation and fibrils connecting *C. albicans* to the cell (Fig. 2b). Figure 3a shows a depression in the plasma membrane where the yeast is perfectly embedded; a fibrillar-floccular layer spread round the cell wall of the *C. albicans* in intimate association with the epithelial cell can be seen (Fig. 3b).

Table 1 shows that the proposed technique of adherence evaluation is reproducible, as there was no statistically significant difference ($P>0.05$) among the three experiments carried out with the 25 fungal isolates. All the yeasts were capable of adhering to the HVEC. However, the mean of adhered *C. albicans* was greater than non-*C. albicans* species (155.5 and 106.1, respectively).

The distribution by species shows that *C. albicans* was the most adherent, followed by *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *Trichosporon* sp., and *Saccharomyces cerevisiae*, respectively (Table 2). The Scheffe test, based on the observed intra-species differences, showed three groupings of similarity (Table 3). However, among the *C. albicans* isolates, a tendency of uniformity was observed with small variations (Fig. 4).

Vaginal yeast isolates seem to prefer adhering to intermediate HVEC. This observation is more evident for *C. albicans*, for which the result was statistically significant. However, the non-*C. albicans* species, which adhered in lower numbers, adhered indiscriminately to intermediate and superficial HVEC (Table 4).

DISCUSSION

In vitro adherence studies of *C. albicans* to oral epithelial cells (OEC) are well established and wide-

Fig. 1. Yeast adherence to human vaginal epithelial cells stained by crystal violet and by the Papanicolaou (Pap) method. **a:** Yeasts stained weakly by Pap only (see arrow). **b:** Yeasts stained by crystal violet and Pap (see arrow); SC (superficial cells); IC (intermediate cells). **c:** 10^7 yeast/mL showing aggregates and overlaying yeasts; **d,e,f:** 10^5 yeast/mL. Amplification = $\times 400$.

spread (17,22,23). However, for HVEC, studies of this type are rare, and consequently, the mechanisms by which the colonization of the vaginal epithelium by yeasts can evolve into VVC and to the recurrent condition (RVVC) are not clear. In this study, a simple methodology capable of assisting studies on the in vitro adherence of yeasts to HVEC (Fig. 1) is proposed.

The proposed assay conditions and the criteria of adherence observed by common optical microscopy were validated by SEM, which confirmed the adherence of the yeasts to the HVEC through their intimate

association (Figs. 2 and 3), as evidenced by the observation of connecting fibrils (Fig. 2b) and the presence of the fibrillar-floccular layer on the yeast (Fig. 3b). The applicability of this technique for studying the adherence of yeasts to HVEC is supported by the reproducibility shown in Table 1.

Various factors can interfere in the in vitro adherence of yeasts to epithelial cells, such as those relating to the cells, to the yeasts, or to environmental factors. The conditions of prior cultivation of the yeast interfere greatly in adherence (24). In this study, a chemically

Fig. 2. Scanning electron micrograph showing several *C. albicans* in intimate association with a human vaginal epithelial cell (a) and in detail (b) showing interdigitation in the areas of contact, with fibrils connecting the yeast to the cell. Amplification = $\times 2,500$.

defined medium YNB, supplemented with 50 mM galactose, was used. According to Kennedy (20), these conditions favor the exhibition of adhesins and other characteristics of the cell surface of *C. albicans* that are highly adhesive to OEC. This fact can be confirmed by the observation of a floccular layer, which stabilizes adhesion and makes it stronger, as seen in Fig. 3b. Other researchers (17,23,25), also recommend this sugar for

promoting the adherence of *C. albicans* to human tissues and plastic surfaces.

The assays were carried out with yeasts in the logarithmic phase of growth (24 hr of pre-cultivation) and 1 hr of co-incubation with the epithelial cells, following Williams et al. (17) and Vidotto et al. (26). In addition to these conditions, Kearns et al. (22) recommended using cells from the same donor. For all

Fig. 3. **a:** Scanning electron micrograph showing a depression of the plasmatic membrane of a human vaginal epithelial cell where a *C. albicans* is perfectly embedded; amplification = $\times 4,500$. **b:** fibrillar-floccular layer spread round the cell wall of the yeast in intimate association with the cell; amplification = $\times 6,000$.

TABLE 1. Average number of yeasts adhered to human vaginal epithelial cells from the three separate experiments, obtained by the simplified technique of adherence evaluation

Experiment	Mean no. <i>C. albicans</i> 600 HVEC $\pm$ SD	Mean no. non- <i>C. albicans</i> 600 HVEC $\pm$ SD	<i>P</i> value
1	133.6 $\pm$ 98.2	116.3 $\pm$ 97.7	<i>P</i> <0.05
2	161.2 $\pm$ 63.9	87.2 $\pm$ 68.8	
3	171.9 $\pm$ 58.3	114.8 $\pm$ 52.6	
Total	155.5 $\pm$ 76.0	106.1 $\pm$ 75.7	

HVEC, human vaginal epithelial cells.

of our experiments, HVEC from one single donor, always collected in the same phase of the menstrual cycle, was used. The ovulation phase was chosen due to the high levels of estrogen, as according to Ferrer (27). VVC is a hormone dependent pathology, or more specifically, estrogen-dependent.

Among the different concentrations of yeasts tested, 5×10^5 yeasts/mL was considered the ideal (Fig. 1d–f) and, for this reason, was the one adopted for the whole study. Lower concentrations were inconsistent and difficult to visualize (data not shown), while higher ones

led to the appearance of yeast aggregates and overlaying (Fig. 1c). This yeast concentration, which gave a proportion of 1:1 in relation to the HVEC, provided adequate consistent readings and good distribution without overlaying, as well as exhibiting well-stained and preserved cells. A greater proportion of microorganisms are recommended by Ghannoum and Radwan (28). However, according to Kennedy (20), elevated concentrations of yeasts tend to co-aggregate themselves, prejudicing interaction with the epithelial cells. This was actually observed in this study (Fig. 1c).

All the isolates of *C. albicans* showed to be strongly adhered to the HVEC (Fig. 4). There was no statistically significant differences in adherence capacity among

TABLE 2. Average number of yeasts adhered to human vaginal epithelial cells according to yeast species

Yeast	Mean no. of yeast 600 HVEC $\pm$ SD*
<i>C. albicans</i>	155.5 $\pm$ 76.0 ^a
<i>C. glabrata</i>	126.2 $\pm$ 81.4 ^b
<i>C. parapsilosis</i>	95.7 $\pm$ 62.0 ^b
<i>C. tropicalis</i>	87.3 $\pm$ 18.0 ^c
<i>Trichosporon</i> sp.	65.7 $\pm$ 68.1 ^{cd}
<i>Saccharomyces cerevisiae</i>	55.5 $\pm$ 29.3 ^d

*Same letters indicate similarity in adhesive capacity ($P > 0.05$) in the Scheffe test of multiple comparisons.
HVEC, human vaginal epithelial cells.

them. It is evident that the property of adherence is isolate-dependent, as suggested by Persi et al. (29) and Ferrer (27). Among non-*C. albicans* had been observed, the strains of this difference was even more significant within the same species.

TABLE 3. Average number of adhered yeasts for each non-*C. albicans* isolate to human vaginal epithelial cells (HVEC)

Number of isolates	Species	Mean no. of yeast 600 HVEC $\pm$ SD*
1	<i>C. glabrata</i>	277.6 $\pm$ 68.6 ^a
2	<i>C. glabrata</i>	179.3 $\pm$ 51.5 ^{ab}
3	<i>C. glabrata</i>	164.2 $\pm$ 40.3 ^{abc}
4	<i>C. glabrata</i>	137.2 $\pm$ 81.6 ^{bc}
5	<i>C. glabrata</i>	95.5 $\pm$ 28.8 ^{bc}
6	<i>C. glabrata</i>	88.8 $\pm$ 19.8 ^{bc}
7	<i>C. glabrata</i>	75.7 $\pm$ 13.5 ^{bc}
8	<i>C. glabrata</i>	70.7 $\pm$ 29.5 ^{bc}
9	<i>C. glabrata</i>	47.3 $\pm$ 18.2 ^{bc}
10	<i>C. tropicalis</i>	87.3 $\pm$ 18.0 ^{bc}
11	<i>C. parapsilosis</i>	126.5 $\pm$ 68.8 ^{bc}
12	<i>C. parapsilosis</i>	64.8 $\pm$ 32.6 ^{bc}
13	<i>Saccharomyces cerevisiae</i>	79.7 $\pm$ 19.5 ^{bc}
14	<i>Saccharomyces cerevisiae</i>	31.3 $\pm$ 12.9 ^c
15	<i>Trichosporon</i> sp.	65.7 $\pm$ 68.1 ^{bc}

*Same letters indicate similarity in adhesive capacity ($P > 0.05$) in the Scheffe test of multiple comparisons.

Fig. 4. Behavior of ten *C. albicans* in adherence “in vitro” study on human vaginal epithelial cells.

TABLE 4. Average number of adhered yeasts according to type of human vaginal epithelial cells, differentiated by Papanicolaou method

Species	Mean no. of yeasts 600 SC $\pm$ SD	Mean no. of yeasts 600 IC $\pm$ SD	P value
<i>C. albicans</i> (n = 10)	117.9 $\pm$ 52.2	193.2 $\pm$ 78.1	<0.05
Non- <i>C. albicans</i> (n = 15)	99.0 $\pm$ 70.9	113.2 $\pm$ 80.5	>0.05
Total (n = 25)	106.5 $\pm$ 64.3	145.2 $\pm$ 88.3	<0.05

SC, superficial human vaginal epithelial cells; IC, intermediate human vaginal epithelial cells.

According to Table 1, significantly more *C. albicans* isolates adhered to HVEC (mean = 155.5) than non-*C. albicans* yeasts (mean = 106.1). This finding reinforces the importance of adherence in the development of VVC and justifies the claim that *C. albicans* is its main agent and, above all, its association with RVVC as shown by Consolaro et al. (4). *C. glabrata*, which is the second main agent of VVC (30), was also the second most adherent (Table 2). However, there was no significant difference between *C. glabrata* and *C. parapsilosis*, *C. tropicalis* and *Trichosporon* sp., or *Trichosporon* sp. and *Saccharomyces cerevisiae*.

S. cerevisiae showed the lowest adherence capacity (Table 2). However, Table 3 suggests great intra-species variability, justifying the disagreements with respect to its involvement in VVC. According to Odds (6) and Posteraro et al. (31), the prevalence of this yeast is low, accounting for less than 1% of cases of VVC. However, it could be greater according to Consolaro et al. (4), who registered its frequency at 5.7%.

Supporting research by Segal et al. (13) and Kalo and Segal (14), the yeasts in general, and especially *C. albicans*, preferred to adhere to IC. Although estrogen is considered the most important hormone in the development of VVC, the aggravation of its symptomatology during the luteal phase may be attributable to the greater capacity of adherence by *C. albicans* to IC. It should also be noted that this does not appear to be the case for non-*C. albicans* yeasts, which adhered well to both of the two cell types (SC and IC).

The accessibility of the proposed methodology, capable of evaluating the adhesive capacity of distinguishable yeasts to SC and IC, will stimulate new studies that need to be carried out in order to consolidate information about the adherence of yeasts to HVEC, their relation with the hormonal influence of the menstrual cycle and, consequently, predisposition to the development of VVC.

In conclusion, as well as being easy to execute and of low cost, the proposed technique has reproducibility, and it is perfectly applicable to the objective of discriminating the adhesive capacity of different *Candida* sp. isolates to the two types of HVEC. With the use of the experimental conditions put forward in this study, the data obtained in different studies can be compared and thus add important information to this fundamental factor of virulence in the development of VVC.

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