Vulvovaginal candidiasis (VVC), specifically its recurrent form, is a highly problematic and a common clinical therapeutic challenge. In clinical practice the diagnosis is mainly based on clinical signs and symptoms and a typical picture apparent on wet mount saline and 10% KOH microscopy. Candida by culture or polymerase chain reaction validates clinical findings but is not routinely obtained or justified. Approximately 10–20% of asymptomatic healthy women harbor culturable Candida sp. and other yeasts in the vagina.

The pathogenesis of acute VVC is currently thought to reflect a microbe imbalance or dysbiosis in the vagina as well as an abnormal host mucosal immune response to the Candida organism. The reasons for and factors involved in the development of dysbiosis are poorly understood. The explanation as to why yeast that normally asymptptomatically colonize the vagina and can cause symptoms and inflammation is controversial. A more recent hypothesis includes a switch from unstructured planktonic yeast growth to biofilm formation that facilitates transition from saprophytic to pathogenic yeast behavior. Potentially, vaginal biofilm could explain acute sporadic VVC or be more relevant in recurrent VVC (RVVC) as a vaginal reservoir for yeast organisms following antifungal therapy and explain vaginal recolonization. Indeed, planktonic and biofilm forms of Candida seem to be different entities. Candida-biofilms have been demonstrated in a variety of experimental conditions in vitro as well as on prosthetic surfaces and endovascular and urethral catheters in vivo.

As a result of growing interest in the topic, numerous investigations have been dedicated to unraveling factors responsible for the development of Candida biofilms. However, despite the widespread belief and broad acceptance of the possibility that Candida biofilms are a critical factor in VVC pathogenesis, we could find no publication that actually demonstrates a microscopic picture of the Candida biofilm on the vaginal surface. The only publication claiming Candida albicans forms biofilms on the vaginal mucosa exclusively includes pictures of smears from vagina without vaginal epithelium. Confirmation of the presence of Candida mucosal biofilm in vivo is thus lacking.

Accordingly, we investigated the histopathology of VVC using fluorescent in situ hybridization (FISH). Biopsies from healthy women, women with bacterial vaginosis (BV), and women with VVC were comparatively investigated using FISH probes specific for fungi and bacteria.

Materials and Methods

Patients

The candidiasis group consisted of 35 randomly selected premenopausal women with confirmed vulvovaginal candidiasis (aged 19–37 years, mean 27 years), 25 women from Guangzhou, China, and 10 women from the...
Why was this study conducted? Biofilms are hypothesized as crucial for the development of vulvovaginal candidiasis (VVC). We investigated vaginal biopsies from 35 women with VVC using fluorescent in situ hybridization and compared with specimens from healthy women and women with bacterial vaginosis.

Key findings Contiguous Candida adherence was not detected in any of the cases or in controls. Histopathological lesions in 26 of 35 biopsies from VVC were exclusively invasive and accompanied by bacterial co-invasion. Lactobacilli (including L. iners and L. crispatus), Gardnerella, and Atopobium were most frequently co-invading.

What does this add to what is known? Polymicrobial mucosal invasion is an unrecognized feature of Candida vaginitis. Our results do not support lactobacilli being beneficial or protective. Different from bacterial vaginosis, we found no biofilm elements in vaginal biopsies obtained from women with VVC.

Friedrichhain Hospital in Berlin, Germany. Five women from Berlin and 8 from China had RVVC, and all others had sporadic VVC.

The diagnosis was based on the clinical appearance and microscopic examination of smears and culture. None of the women received antifungal treatment for 2 months prior to the investigation. In the candidiasis group, FISH-performing researches were not blinded to group diagnosis, but the investigators were not aware of individual data including results of culture, swab investigations, and clinical course.

Material from paraffin-embedded biopsies from 25 healthy women (aged 20–35 years, mean 26.4 years) investigated for routine care and 30 women with bacterial vaginosis (aged 18–40 years, mean 27.2 years) served as controls. All women in control groups were premenopausal. These materials had been preserved from previous studies on bacterial vaginosis (BV) and described.14,15

Biopsies of about 3–5 mm diameter were taken from the middle side wall of the vagina with biopsy forceps (No. ER 058 R; Schubert, Aesculap, Tuttlingen, Germany) and fixated with modified nonaqueous Carnoy solution (6/1/2 volume ethanol/glacial acetic acid/chloroform). The fixated materials could be stored in Carnoy at room temperature for up to 6 months. Usually the time used was convenient for the investigated subject and laboratory staff. Carnoy-fixed material was processed and embedded into paraffin blocks using standard techniques.

Four-micrometer-thick sections were placed on Super Frost plus slides (R. Langenbrinck, Emmendingen, Germany). Sections of vaginal biopsies were hybridized with ribosomal RNA-based FISH probes specific for all bacteria, Gardnerrla cluster, Atopobium cluster, lactobacilli, Lactobacillus iners and Lactobacillus crispatus, all yeasts, and Candida albicans (Table 1).

All hybridizations were performed at 50°C using a protocol described previously.16 A Nikon e600 fluorescence microscope was used. The images were photodocumented with a Nikon Dxm 1200F color camera and software (Nikon, Tokyo, Japan).

The study was reviewed and approved by the Institutional Review Board of Jinan University.

Results Hybridization signals positive for yeasts were detected in biopsies from 26 of 35 women with VVC/RVVC. No yeasts were observed in the healthy group and the BV group, regardless of the protocol used (Figure 1).

Within the candidiasis group, signals were positive for Candida albicans in 18 women, and 8 further samples were negative for Candida albicans but were hybridized with the universal for most yeasts PF2 probe (Table 2 and Figures 2–6). The histological patterns of candidiasis were identical in both sporadic as recurrent VVC.

We found no yeast in the biopsies of 9 women from the VVC group (5 from China and 3 from Berlin). This could

TABLE 1
Applied FISH probes

<table>
<thead>
<tr>
<th>Probe name</th>
<th>Sequence 5’–3’</th>
<th>Target organism</th>
<th>RNA values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eub 338</td>
<td>GCT GCC TCC CGT AGG AGT</td>
<td>Most bacteria</td>
<td>16S rRNA</td>
</tr>
<tr>
<td>Gard 662</td>
<td>CCA CGG TTA CAC CGC GAA</td>
<td>Gardnerella cluster</td>
<td>16S rRNA</td>
</tr>
<tr>
<td>Liner23-2</td>
<td>CTG CTC ACC TAS TTT CGG G</td>
<td>Lactobacillus iners</td>
<td>23S rRNA</td>
</tr>
<tr>
<td>Lab158</td>
<td>GGT ATT AGC A(T/C)C TGT TTC CA</td>
<td>Lactobacillus sp Enterococcus sp</td>
<td>16S rRNA</td>
</tr>
<tr>
<td>Lcrisp16-1</td>
<td>GCT CAT TCG AAT AGA AAT CTG T</td>
<td>Lactobacillus crispatus</td>
<td>16S rRNA</td>
</tr>
<tr>
<td>Ato291</td>
<td>GGT CGG TCT CTC AAC CC</td>
<td>Atopobium cluster</td>
<td>16S rRNA</td>
</tr>
<tr>
<td>Fungi</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caal</td>
<td>GCC AAG GGT TAT ACT CGC T</td>
<td>Candida albicans</td>
<td>18S rRNA</td>
</tr>
<tr>
<td>PF2</td>
<td>CTC TGG CTT CAC CCT ATT C</td>
<td>Most yeasts</td>
<td>18S rRNA</td>
</tr>
</tbody>
</table>

rRNA, ribosomal RNA.

be due to the nonuniform fungal invasion over the epithelial surface, with some regions remaining uninvolved as demonstrated in Figure 3 (a1—a3).

All fungal cells detected by hybridization were mainly invasive, with variable hyphae penetrating more or less deeply into the epithelial surface of the biopsy (Figures 2—6) and leaving some of the biopsy areas completely free (Figure 3 [a1—a3]. The invasive character of the infection was especially well seen when fungal fluorescence hybridization was 4′,6′-diamino-2-phenylindole (DAPI) counterstained, revealing all eukaryotic cells (Figures 2—6).

Occasionally single fungal cells or blastospores could be seen in slime-covering biopsies; however, a fungal biofilm embedded in its own extracellular matrix was never observed.

Fungal infiltration was always accompanied by co-invasion with bacterial components. Bacteria were either evenly distributed over the depth of the fungal invasion (typical for Gardnerella and some lactobacilli, 18 cases total) or concentrated at the forefront of the fungal invasion (Lactobacillus iners co-invasion, Figure 5 and 6, 8 cases total).

Co-invading bacteria were polymicrobial, representing a broad spectrum of vaginal microbiota. Lactobacilli, Gardnerella, and Atopobium were most frequently seen. Gardnerella was always associated either with considerable amounts of lactobacilli ranging from 10^8 to 10^{11} (80%) and/or Atopobium (60%). A high concentration of lactobacilli could accompany the fungal invasion in the absence of Gardnerella or Atopobium.

(Figures 5 and 6).

Co-infiltrating lactobacilli were often but not exclusively represented by Lactobacillus iners (Table 1).

**Comment**

Many excellent reviews are dedicated to different aspects of Candida research.2—5,7,8,12 Our perception of VVC is based only on interpretation of indirect indices: epidemiology of

<table>
<thead>
<tr>
<th>FIGURE 1</th>
<th>Mucosa and biofilms</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong></td>
<td>Biopsy from a healthy woman, Eub 338 Cy3 × 400, (all bacteria, yellow fluorescence). The surface of the healthy vaginal epithelium is free from adherent bacteria. <strong>B</strong></td>
</tr>
</tbody>
</table>


**TABLE 2**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Superficial biofilms</th>
<th>Infiltration by fungi plus bacteria</th>
<th>Nonadherent microorganisms in slime only</th>
<th>Candida plus other fungi</th>
<th>Gard 662 Gardnerella</th>
<th>Ato 291 Atopobium</th>
<th>Lab 158 (without Gard or Ato)</th>
<th>L. iners</th>
</tr>
</thead>
<tbody>
<tr>
<td>WC (n = 35)</td>
<td>0</td>
<td>26^a</td>
<td>2</td>
<td>18/8</td>
<td>10</td>
<td>6</td>
<td>19 (9)</td>
<td>11</td>
</tr>
<tr>
<td>BV (n = 30)</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>30</td>
<td>25</td>
<td>21 (0)</td>
<td>12</td>
</tr>
<tr>
<td>Healthy (n = 25)</td>
<td>1</td>
<td>0</td>
<td>9</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>9 (8)</td>
<td>3</td>
</tr>
</tbody>
</table>

^a The fungal invasion was always accompanied by bacterial coinfiltration as detected with the Eub 338 FISH probe, which represents all bacteria. Such a bacterial coinfiltration is characteristic for VVC and never occurred in healthy women or in BV. Gardnerella, even when highly concentrated, accompanied Candida in WC but did not build a characteristic biofilm as seen in BV.


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colonization and disease, macroscopic appearance of lesions, symptoms, investigation of vaginal smears using microscopy, culture or molecular genetic identification of microorganisms, and simulation of infection/colonization in vitro and in experimental animal models.

This is the first study that directly visualizes Candida microorganisms in vaginal biopsies, and the results substantially contradict widespread assumptions. We found no biofilm elements in vaginal biopsies obtained from women with VVC. Results were identical in women with acute sporadic or recurrent VVC.

This is in contrast to biofilms seen in bacterial vaginosis. Moreover, Candida cells on the surface were single, non-confluent, and what is most important nonadherent to the vaginal epithelium. This cannot be explained by processing sample biases.

Carnoy solution, which we used for fixation of the biopsy samples, demonstrated its high efficiency in preserving biofilms and even bacteria-free slime on different mucosal surfaces. However, in appropriate and representative specimens obtained in VVC, we were unable to detect any contiguous Candida layer on the mucosal or epithelial surface. It is possible that the cementing properties of the extracellular matrix, which is produced by Candida, is very low, unable to maintain Candida attachment to the vaginal surface and not different from those produced by any

**FIGURE 2**
Examples of Candida invasion

Biopsies from 5 women with vulvovaginal Candidiasis Caal Cy3 (Candida, yellow fluorescence) demonstrating different grades of infiltration of the vaginal epithelium by Candida without adjacent adherent biofilms or components. The infiltration is particularly well seen by counterstaining of the vaginal tissues with DAPI (blue fluorescence of the human tissue). Magnification, ×1000 (A); ×400 (B, D, and E); ×100 (C).

DAPI, 4',6'-diamino-2-phenylindole.

other microorganisms growing in a colony on a culture plate.

The fixation or adherence of Candida microorganisms to the vaginal surface is possible or likely through hyphal mucosal invasion. Visually apparent white membranes, which cover the vaginal epithelium in vulvovaginal candidiasis and appear to be biofilms, are actually deep inflammatory infiltrates.

In the past, few studies have included vaginal biopsies in women with VVC. In the absence of such critical tissue status information, vaginal candidiasis has been considered as entirely superficial mucosal infection and the lack of even superficial invasion emphasized. In fact, some clinicians refer to Candida vaginitis as infection of vaginal secretions only. Moreover, the histological appearance of nonvaginal Candida lesions presented in some publications are all definitively invasive (Gow and Hube, \textsuperscript{17} page 407, Figure 1, A, B, and C, and Figure 2; Jabra-Rizk et al. \textsuperscript{18} page 2729, Figure 9, B and C; Gao et al. \textsuperscript{6} page 737, Figure 9; Allison et al. \textsuperscript{7} page 8, Figure 6) and similar to our findings.

A most prominent feature in our study is a deep infiltration of vaginal tissue. The infiltration is not homogeneous with some regions affected more
heavily than others and some remaining completely free. The removal of such lesions is impossible without stripping the integrity of the epithelial layer. In spite of the depth of infiltration, it is not full thickness, and disseminated infection is not reported clinically.

Candida colonization and infection occur in polymicrobial environments. Our findings suggest possible bacteria-yeast interactions in tissue invasion. With regard to VVC, Gardnerella spp. was claimed to promote and lactobacilli to supress Candida biofilms.\(^7,19\) While our data support a contributory promoting role for Gardnerella, little was forthcoming to support lactobacilli being beneficial or protective.

On the contrary, lactobacilli were visualized accompanying Candida infection even more often than Gardnerella. The concentrations or density of lactobacilli within the vaginal epithelium was high, both for L. iners and other Lactobacillus species including L. crispatus. Because the lactobacilli were homogeneously distributed within the lesion and in some cases even enriched at the forefront of Candida infiltration, a pathogenic fungal-bacterial symbiosis seems more likely than a secondary saprophytic relationship.

Obviously pathogenic consortia include a variety of different types of bacteria as demonstrated in polymicrobial BV biofilms. The interaction between fungi and bacteria may contribute to the switch from saprophytic to invasive forms of fungal

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**FIGURE 4**

*Candida-Gardnerella co-invasion*

Biopsy from a woman with VVC, multicolor hybridization with a mix of probes Caal Cy3 (*Candida*, yellow fluorescence [A]) and Gard Cy5 (*Gardnerella*, dark red [A and B]) ×1000 and counterstained with DAPI (blue fluorescence showing all rich on DNA stuctures [B]), on the right side. Similar to the observations in bacterial vaginosis, Gardnerella can be demonstrated in VVC in high numbers in association with *Candida* (panel A) and a high density of Gardnerella proliferation resembling a biofilm formation similar to that observed in BV, shown in Figure 1B. However, the DAPI counterstain demonstrates clearly that in VVC Gardnerella is no longer only adherently attached but invasive and located below the epithelial surface (panel B).

*BV*, bacterial vaginosis; *DAPI*, 4',6'-diamino-2-phenylindole; *VVC*, vulvovaginal candidiasis.

growth. Quorum-sensing research investigates stimuli that synchronize a correlated response of the microorganism to population density. Presently it is mainly focused on cross talking within single groups of microorganisms,\textsuperscript{5,9,10} this should be widened to polymicrobial populations.

In our series, 26% of the lesions did not hybridize with the \textit{Candida albicans}-specific Caal FISH probe but were positive with the universal yeast probe. At present, we cannot designate the origin of these fungi. Either the Caal FISH probe hybridizes less stringently to other representatives of \textit{Candida}, or the infiltration is induced by non-\textit{Candida} yeasts in some cases, which were clinically diagnosed as VVC. However, the extent of the infiltration by \textit{Candida albicans} and other yeasts was identical, even in the absence of hyphae (Figure 6), indicating similar pathogenic potential.

Our data point to vaginal epithelial surface or mucosal invasion as an unrecognized feature of VVC. Although vaginal biopsies were not obtained in asymptomatic women without VVC but colonized with \textit{Candida}, it is likely that such common saprophytic colonization occurs in the absence of any tissue invasion. Most importantly, no evidence of in situ, in vivo \textit{Candida} biofilm existence emerged.

The clinical relevance of these findings is largely unknown, but the absence of finding a biofilm in VVC implies that antibiofilm therapy is not indicated because it may be required in BV. The finding of tissue invasion in VVC may have relevance to recolonization of vagina with candida organisms following antifungal therapy (i.e., the vaginal mucosa may serve as organismo reservoir to explain re-colonization following treatment\textsuperscript{20}).

Acknowledgments

The work has not been published previously and is not under consideration for publication elsewhere. The publication is approved by all authors, and tacitly or explicitly by the responsible authorities in which the work was carried out, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright holder. Each author named in the byline participated actively and sufficiently in the study reported. The author contributions included the following: Drs Swidsinski, Guschin, XY, and Tertychny designed the study; Drs Dörfel, Tang, Tertychny, Luo, and Jiang conducted the study; Drs Sobel and Verstraeten critically revised the manuscript; Drs Swidsinski, Guschin, and Dörfel performed the fluorescent in situ hybridization; and Drs Tertychny, Tang, Luo, and Jiang analyzed the data. All authors contributed to the conception of the work, revising of the data, shaping of the manuscript, and approved the final draft submitted.

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