Adherent Biofilms in Bacterial Vaginosis

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OBJECTIVE: Bacterial vaginosis is a common infectious disorder. Although known since ancient times, little progress has occurred in identifying causal factors. Our aims were to study the bacterial community structure and the spatial organization of microbiota on the epithelial surfaces of vaginal biopsy specimens.

METHODS: We investigated the composition and spatial organization of bacteria associated with the vaginal epithelium in biopsy specimens from 20 patients with bacterial vaginosis and 40 normal premenopausal and postmenopausal controls using a broad range of fluorescent bacterial group-specific rRNA-targeted oligonucleotide probes.

RESULTS: Bacterial vaginosis was associated with greater occurrence and higher concentrations of a variety of bacterial groups. However, only *Gardnerella vaginalis* developed a characteristic adherent biofilm that was specific for bacterial vaginosis.

CONCLUSION: A biofilm comprised of confluent *G vaginalis* with other bacterial groups incorporated in the adherent layer is a prominent feature of bacterial vaginosis.
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LEVEL OF EVIDENCE: II-2

It was originally postulated by Gardner and Dukes in 1955 that *Haemophilus vaginalis*, now known as *Gardnerella vaginalis* and affiliated to the family *Bifidobacteriaceae*,[1] was the sole cause of bacterial vaginosis, because *G vaginalis* was recovered from the vaginas of 92% of patients with bacterial vaginosis and from no woman without bacterial vaginosis.[2] However, the role of *G vaginalis* in bacterial vaginosis became less clear as investigators began to report a 30 to 40% prevalence of *G vaginalis* among normal women. In addition, bacteria other than *Gardnerella* were positively associated with vaginosis,[3] and evidence of a possible endogenous source of bacteria due to anal–vaginal transfer emerged.[4]

Bacterial vaginosis is currently considered to be a synergistic polymicrobial syndrome that is characterized by depletion of *Lactobacillus* spp., especially those that produce hydrogen peroxide, and an intense increase (100- to 1000-fold above normal levels) in the quantity of commensal vaginal anaerobic bacteria, including *G vaginalis*, *Prevotella* spp, anaerobic gram-positive cocci, *Mobiluncus* spp, *Mycoplasma hominis*, and *Atopobium vaginalis*.[1,3] Enzymes and decarboxylases produced by these anaerobic bacteria are thought to degrade proteins and convert the amino acids to amines. These amines raise the vaginal pH and produce a characteristic fishy odor. The diagnosis of bacterial vaginosis is made primarily clinically, based on the Amsel criteria: a vaginal pH more than 4.5, a characteristic milky discharge, a positive "whiff test," and the presence of bacteria-coated epithelial cells termed "clue cells" in a wet mount preparation.[5,6] Polymicrobial involvement in bacterial vaginosis is now generally accepted and has been confirmed by culture, microscopic, and molecular genetic methods.[7,8] The shift in the composition of vaginal bacterial communities has been extensively studied by investigation of vaginal secretions and smears. However, little is known about how the different bacterial groups implicated in bacterial vaginosis interact with the vaginal epithelial surface.

Our aims were to study the bacterial community structure and the spatial organization of microbiota on the epithelial surfaces of vaginal biopsy specimens using fluorescence in situ hybridization with a broad range of bacterial group-specific rRNA-targeted probes.

MATERIALS AND METHODS

Twenty women with bacterial vaginosis and 40 controls (20 premenopausal and 20 postmenopausal women)

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who attended the Vivantes Clinics for Gynecology as inpatients or outpatients from August 2004 to April 2005 were enrolled. All women were white and selected randomly. All subjects gave informed consent according to the protocol approved by the Ethics Commission of the Charité Hospital. The diagnosis of bacterial vaginosis was based upon the presence of a vaginal discharge with 3 of the 4 following characteristics: a homogeneous appearance, a pH of more than 4.5, a fishy amine odor upon the addition of 10% potassium hydroxide, and the presence of clue cells. A vaginal Gram stain was prepared in 7 of 20 patients in whom the bacterial vaginosis was diagnosed for the first time. Thirteen women had recurrent disease with 1 to 4 episodes of bacterial vaginosis treated in the same gynecologic office. Women were excluded from the study if they had received systemic antibiotic therapy or local vaginal antimicrobial therapy within the preceding 2 months, were menstruating at the time of the examination, were using an intrauterine contraceptive device, or had a history of sexually transmitted disease.

Culturing of vaginal flora was performed semiquantitatively. Cotton swabs were used to obtain samplings from the lateral vaginal wall and placed into Amies transport medium (transystem, HAIN Lifescience, Nehren, Germany). Samples were plated on a culture medium and placed in either an anaerobic or a 5% CO_2 atmosphere at 36°C within 2 hours after collection. Schaedler/KV agar, V agar (for *G vaginalis*), Columbia blood agar, MacConkey II agar, and CHROMagar *Candida* (Becton Dickinson and Company, Franklin Lakes, NJ) were used. *Gardnerella vaginalis* was identified on the basis of β hemolysis on *Gardnerella* agar, negative catalase reaction, and Gram stain. *Lactobacilli* were identified to the genus level by Gram stain and colony morphology and negative catalase test. Anaerobic gram-negative rods were identified by using commercial biochemical tests API ID 32A, aerobic bacteria by using API 20 E, API ID 32 Strep, and API Coryne galleries (bio Merieux, La Balme-les-Grottes, Montalieu-Vercieu, France).

Biopsy specimens (3–5 mm diameter) for fluorescence in situ hybridization analysis were taken from the middle side wall of the vagina with biopsy forceps (Nr. ER 058 R, Aesculap, Tüttlingen, Germany), fixed in nonaqueous Carnoy solution[9] for 2 hours, and then processed and embedded into paraffin blocks using standard techniques. Four-micrometer sections were placed on SuperFrost slides (R. Langenbrinck, Emmendingen, Germany) for fluorescence in situ hybridization studies.

Oligonucleotide probes were synthesized with a carbocyanine dye (Cy3), fluorescein isothiocyanate or Cy5 fluorescent dye at the 5′ end (MWG Biotech, Ebersberg, Germany). A broad range of domain-, group-, and species-specific fluorescence in situ hybridization probes appropriate for the expected anogenital origin of the bacteria were applied (Table 1). Bacteria were detected by fluorescence in situ hybridization and 4',6-diamidino-2-phenylindole counter stain. The 4',6-diamidino-2-phenylindole counter stain visualizes the DNA of bacteria, fungi, and host cells (blue fluorescence). A Nikon e600 fluorescence microscope (Nikon, Tokyo, Japan) was used. The data were photographically documented with a Nikon DXM1200 camera and software (Nikon). High-power (x1000 magnification) photographs were made consecutively from the entire specimen surface. Bacteria were quantified separately for the area lining intact epithelium and for the subepithelial surface that was artificially exposed by trauma with the biopsy forceps. The quantification of bacteria was based on the assumption that a 10-μL sample with a cell concentration of 10^7 cells per milliliter results in 40 cells per average microscopic field at magnification of 1,000.[9]

Single bacteria irregularly scattered over the epithelial surface were defined as no biofilm. Conglomerates of bacteria loosely attached to the epithelial surface without any perceivable structural organization or stable composition were defined as unstructured biofilms. Lawns of bacteria adherent to the epithelial surface and mainly composed of specific bacterial groups were defined as adherent bacterial biofilms. For convenience, we often use the word biofilm, meaning in each case bacterial biofilm. Neither fungal biofilms nor host secretions, which also can be regarded as "biofilm," were investigated.

The autofluorescence background of the human tissue allowed tissue structures to be seen during the fluorescence in situ hybridization studies. Nevertheless, for each biopsy at least 1 or 2 additional stains (hematoxylin and eosin or methenamine silver or Gram stain or all 3) were performed. The methenamine silver stain and the Brown-Hopps modification of the Gram stain were performed as described.[32]

For ultrastructural evaluation, parts of the paraffin-embedded samples were removed from the blocks, and reprocessed by xylol immersion and subsequent rehydration in decreasing methanol concentrations to buffer. Further processing was achieved by postfixation in 1% osmium tetroxide, dehydration through graded methanols, penetration, and embedding in epoxy resin. Using a diamond knife, ultrathin sections were cut from the resin blocks with an LKB ultramicrotome (Ultrotome NOVA; LKB, Broma, Sweden), mounted on 100-mesh copper grids, stained

Table 1. Fluorescence In Situ Hybridization Probes*

Name	Target	Reference
Non338	Nonsense probe used to test for nonspecific binding	Manz et al[10]
Eub338	Virtually all bacteria, Kingdom (*Eu) Bacteria*	Amann et al[11]
HGC	*Actinobacteria* (*Gardnerella, Mobiluncus, Atopobium,* etc)	Roller at al[12]
Ato291	*Atopobium, Coriobacterium, Eggerthella and Collinsella* spp	Harmsen et al[13]
Cor653	*Coriobacterium* group	Harmsen et al[13]
Bif164, 662	*Bifidobacteriaceae*, including *Gardnerella vaginalis* (1 mismatch)	Langendijk[14]
GardV	Probe derived from Bif662 with 0 mismatches to *G vaginalis*	Present study†
Bif1278	*Bifidobacterium* (4 mismatches to *G vaginalis*)	Langendijk[14]
LGC	*Firmicutes* (*Lactobacilli, Streptococci, Staphylococci, Mycoplasma* etc.*)*	Meier et al[15]
Lab158	*Lactobacillus* and *Enterococcus*	Harmsen et al[16]
Bac303	*Bacteroides/Prevotella*	Manz et al[17]
Bdis656	*Bacteroides distasonis*	Franks et al[18]
Ebac	*Enterobacteriaceae*	Bohnert et al[19]
Ec1531	*Escherichia coli*	Poulsen et al[20]
Y16s-69	*Yersinia*	Trebesius et al[21]
Arc1430	*Arcobacter*, epsilon subclass of *Proteobacteria*	Snaidr et al[22]
Aer66	*Aeromonas*	Kämpfer et al[23]
Erec482	*Clostridium coccoides–Eubacterium rectale* group	Franks et al[18]
Phasco	*Phascolarctobacterium faecium*	Harmsen et al[24]
Chis150	*Clostridium histolyticum*	Franks et al[18]
Clit135	*Clostridium lituseburense*	Franks et al[18]
Lach	Subgroup of Erec (including *Lachnospira multipara*)	Harmsen et al[24]
Enc131	*Enterococcus*	Frahm et al[25]
Strc493	*Streptococcus*	Franks et al[18]
Efaec	*Enterococcus faecalis, Enterococcus sulfuricus*	Jansen et al[26]
Veil	*Veillonella*	Harmsen et al[24]
Rbro, Rfla	*Ruminococcus bromii, Ruminococcus flavefaciens*	Harmsen et al[24]
UroA, UroB	*Ruminococcus obeum*-like bacteria (subgroup of Erec)	Zoetendal et al[27]
Ecyl	*Eubacterium cylindroides*	Harmsen et al[24]
Ehal	Subgroup of Erec (including *Eubacterium hallii*)	Harmsen et al[24]
Ser1410	Genus *Brachyspira*	Jensen et al[28]
CF319a	*Cytophaga–Flavobacteria* group	Manz et al[17]
Fprau	*Fusobacterium prausnitzii* group	Suau et al[29]
Hpy-1	*Helicobacter pylori* epsilon subclass of *Proteobacteria*	Feydt-Schmidt et al[30]
Sfb	*Segmented filamentous bacteria*	Urdaci et al[31]

* Formamide concentration and hybridization temperature were chosen to achieve the optimal stringency as described in the references. Additional hybridizations using a permeation step with lysozyme for 15, 30 and 60 minutes were performed in parallel for detection of gram-positive bacteria.

† GardV probe sequence is 5´- CCA CCG TTA CAC CGC GAA- 3´. Fluorescence in situ hybridization is performed using a washing temperature of 50°C and no formamide.

with uranyl acetate and lead citrate, and examined with a Zeiss EM 10 transmission electron microscope (Carl Zeiss, Oberkochen, Germany) at 60 kV.

Mean values and standard deviations were calculated from the bacterial counts. Using analysis of variance and χ^2 test, a P value of less than .05 was considered significant.

RESULTS

The mean age and range of the 20 bacterial vaginosis patients was 27.3 years (range 8 to 40 years), of the 20 premenopausal women was 26.1 years (range 18 to 43 years), and of the 20 postmenopausal women was 57.3 years (range 42 to 81 years). The types and concentrations of bacteria that were isolated from vaginal cultures of patients with bacterial vaginosis or control premenopausal and postmenopausal patients are summarized in Table 2. *Lactobacilli* were commonly cultured in high concentrations from premenopausal control patients, but were less frequently cultured from patients with bacterial vaginosis and from postmenopausal controls. *Gardnerella vaginalis* was more frequently identified in vaginal cultures from patients with bacterial vaginosis (19/20 patients), compared with premenopausal (3/20) and postmenopausal controls (2/20). Furthermore, vaginal *G vaginalis* concentrations of 10^5 CFU/mL or more were only detected in patients with bacterial vaginosis (16/20 patients compared with 0/40 controls; $P <$ .001). The incidence of *Candida* was similar in bacte-

Table 2. Types and Concentrations of Bacteria Isolated From Vaginal Culture

Type of Bacteria and CFU/mL	Bacterial Vaginosis (n = 20)	Premenopausal Controls (n = 20)	Postmenopausal Controls (n = 20)
Lactobacilli $<10^2$	6	5	3
Lactobacilli $10^{3–4}$	2	3	1
Lactobacilli $10^{5–7}$	3	4	5
Lactobacilli $>10^8$	0	3	1
Gardnerella vaginalis $<10^2$	1	3	1
G vaginalis $10^{3–4}$	2	0	1
G vaginalis $10^{5–7}$	5	0	0
G vaginalis $>10^8$	11	0	0
Candida $<10^2$	3	4	2
Candida $10^{3–4}$	0	1	2
Candida $>10^5$	2	0	3
Corynebacteria	4	1	2
Mobiluncus	1	0	0
Streptococci/Enterococci	5	2	3
Staphylococci	8	5	4
Enterobacteriaceae (*Escherichia coli*, *Proteus* etc.)	7	2	5
Bacteroides/Prevotella	9	3	5
Clostridia	2	1	2
Other anaerobes	8	2	3
Other aerobes	3	1	3

rial vaginosis patients and controls. Aerobic and anaerobic bacteria were more frequently isolated from patients with bacterial vaginosis compared with controls (Table 2).

Using fluorescence in situ hybridization, no biofilm could be detected in 14 of 20 healthy premenopausal and 6 of 20 postmenopausal controls. In these patients, only single bacteria contacted the epithelium, corresponding to concentrations of less than 10^6 bacteria per milliliter (Table 3). Long rods that positively hybridized with the Lab probe (*Lactobacillus*) were dispersed over the epithelial surface, with no bacteria seen in most of the microscopic fields and 1 to 3 bacteria in a few fields (Fig. 1). In all but 2 of these patients, no other bacterial groups could be detected by fluorescence in situ hybridization. In 1 woman, single bacteria that hybridized with either Bac (*Bacteroides*) or GardV (*Gardnerella*) probes were involved. In the other, single Ato-positive (*Atopobium*) bacteria were found additionally to Lab-positive (*Lactobacillus*) bacteria. Bacteria were observed equally often on the intact epithelial surface and on the tissue exposed due to biopsy tear.

A loose bacterial biofilm without apparent structure was observed in 11 healthy postmenopausal women, 5 premenopausal women, and 2 women with bacterial vaginosis (Fig. 2). This biofilm was mainly composed of bacteria that positively hybridized with the Lab (*Lactobacillus*) probe in concentrations between 10^7 and 10^{10} bacteria per milliliter. Other bacterial groups were present in secretions, irregularly intermixed with the Lab-positive (*Lactobacillus*) bacteria and partially contacting the epithelial surface. Despite this contact, the observed biofilm was probably nonadherent, because similarly organized clouds of bacteria were observed on the sheared subepithelial portions of the biopsy. The proportion of bacterial groups other than those hybridizing with the Lab (*Lactobacillus*) probe was low within this biofilm, and represented less than 1% for each organism and less than 10% of the biofilm for all organisms combined.

A dense bacterial biofilm was attached to at least 50% of the intact epithelial surface in 90% of the biopsy specimens from subjects with bacterial vaginosis and 10% of the normal controls ($P < .001$). The biofilm was highly characteristic and unmistakable in appearance. Bacteria were nearly homogeneously composed of stacked short rods with almost no free spaces between single cells and the epithelial surface. Bacterial concentrations within this biofilm reached 10^{11} bacteria per milliliter (Fig. 3). Highly concentrated biofilm covered almost the entire intact epithelial surface in 8 patients with bacterial vaginosis. The biofilm was patchy in 14 other subjects (10 patients with bacterial vaginosis, 1 premenopausal woman, and 3 postmenopausal women), with islands of high concentration of bacteria alternating with regions covered with single bacteria or small bacterial groups. The adherent biofilm was absent on the torn surfaces of the biopsy.

This adherent biofilm was mainly composed of 3

Table 3. Maximal and Mean Concentrations of Bacteria Lining the Epithelial Surface of the Vagina and the Proportion of Bacteria That Hybridized With Different Group- and Species-Specific Probes

Group	Appearance of the Biofilm (n)	Bacterial Concentrations Within Single Patients x 10^{10} Range* Maximum	Mean ± SD	Probe Name GardV A	GardV B	Ato A	Ato B	LGC/ Lab A	LGC/ Lab B	Cor A	Cor B	Bac A	Veil A	Str A	Rbro A	Ehal A	Clit A
BV	Adherent (18)	0.8–20	3.7 ± 3.3	18	70–90	16	1–30	17	0.1–3	1	15	7	4	6	2	2	1
	Unstructured (2)	0–1.2	0.1–0.9	1	< 0.1	0	0	2	90	0	0	0	0	1	0	0	0
	None	–	–														
Pre M	Adherent (1)	0.1	0.1	1	70–90	0	0	1	1–3	0	0	0	0	0	0	0	0
	Unstructured (5)	0–1.3	0.13 ± 0.15	3	0.1–1	2	> 0.1	5	90	1	0.1	1	1	1	0	0	0
	None (14)	–	< .005	0	0	0	0	14	100	0	0	1	0	0	0	0	0
Post M	Adherent (3)	0.4–2.0	1.2	3	75–99	1	14	3	1–3	0	0	1	0	0	0	0	0
	Unstructured (11)	0–1.0	0.08 ± 0.25	6	0.1–1	3	> 0.1	11	90	1	0.1	3	2	3	1	0	0
	None (6)	–	< .005	1	< 0.1	0	0	6	100	0	0	0	0	0	0	0	0

SD, standard deviation; A, number of patients with bacteria detected by a group-specific probe; B, percentage of bacteria hybridizing with a single probe of the whole biofilm; BV, bacterial vaginosis; Pre M, premenopausal; Post M, postmenopausal.

* Maximal concentrations were enumerated within 1 patient at the site of the maximal density of the biofilm covering at least 10% of the intact vaginal mucosa. Mean concentrations were enumerated for the whole epithelial surface of a single biopsy. The mean concentrations of bacteria within the unstructured biofilm of both control groups differed significantly from the mean concentrations of bacteria within adherent biofilm in the group with bacterial vaginosis ($P < .001$). The occurrence of high concentrated GardV (*Gardnerella*)– and Ato (*Atopobium*)–positive bacteria was significantly higher in the bacterial vaginosis group than in both control groups ($P < .001$). The occurrence and the absolute concentrations of the Lab-positive (*Lactobacillus*) bacteria did not differ significantly among all groups. The occurrence of Bac (*Bacteroides*)–, Veil (*Veillonella*)–, Str (*Streptococcus*)–, Rbro (*Ruminococcus*)–, and Clit (*Clostridium lituseburense*)–positive bacteria was higher in the bacterial vaginosis group than in both controls groups ($P < .05$).

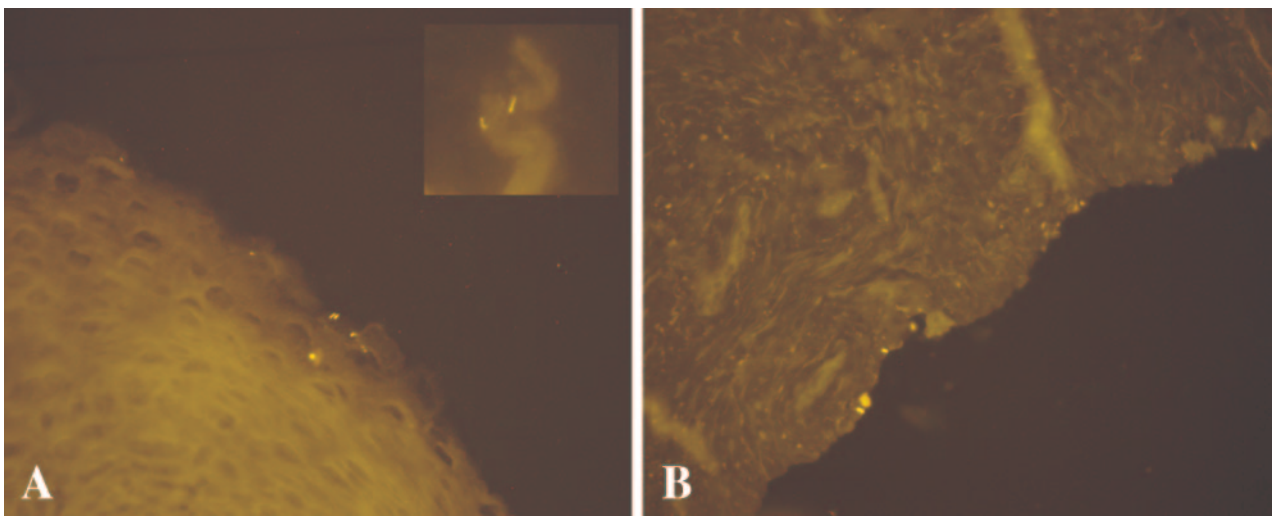

Fig. 1. Vaginal epithelium from a healthy premenopausal woman hybridized with a universal probe (x400) and Lab (*Lactobacillus*) probe (inset x1000). Only a small number of Eub/Lab-positive bacteria (All bacteria/*Lactobacillus*) are scattered over the surface of the intact epithelium **(A)**. Long rods can be perceived with high magnification (inset). Bacteria are found in similar concentrations on the subepithelial surface of the biopsy that was exposed by the mechanical trauma of the tissue **(B)**. Although the epithelial tissue structures are not stained, they are well visualized due to autofluorescence.
Swidsinski. Biofilms in Bacterial Vaginosis. Obstet Gynecol 2005.

bacterial groups. Bacteria that positively hybridized with GardV probe (*Gardnerella*) composed 60% to 95% of the biofilm mass. Bacteria that positively hybridized with the Ato probe (*Atopobium*) were homogeneously intermixed with the GardV-positive (*Gardnerella*) biofilm in 70% of the biopsy specimens and composed 1% to 40% of the biofilm mass. Bacteria that positively hybridized with the Lab (*Lactobacillus*) probe were found in all but 1

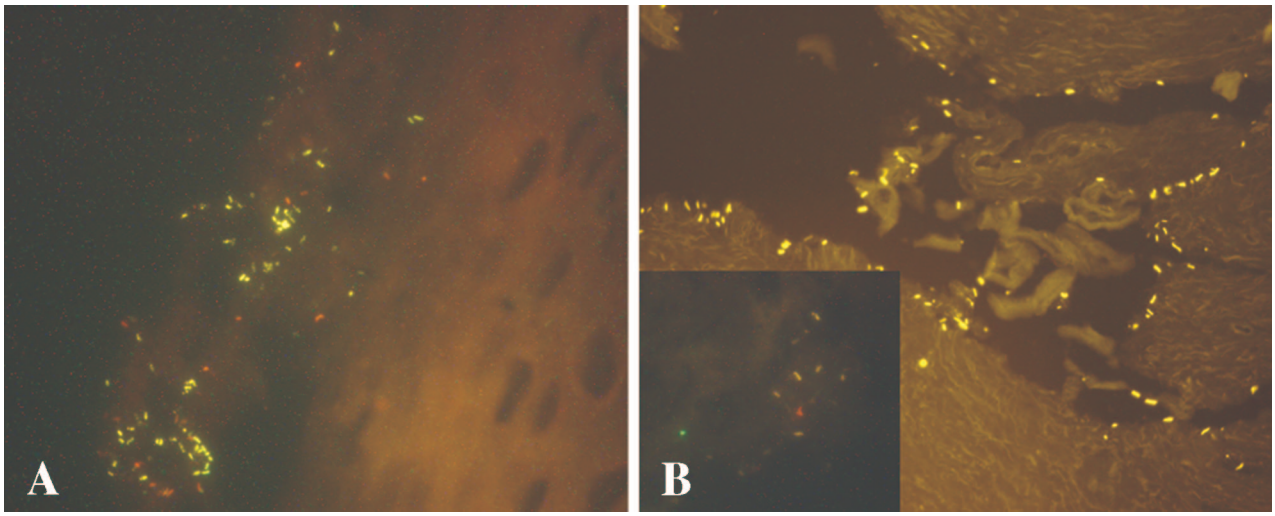

Fig. 2. Vaginal epithelium from healthy postmenopausal woman with unstructured biofilm. A triple contrasted hybridization with Lab-Cy3 (orange), Eub-fluorescein isothiocyanate (green), and GardV-Cy5 (red) probes is shown on the left **(A)**, x1000 magnification. Different bacterial groups are mixed and loosely attached to the epithelium suspended within secretions. *Gardnerella vaginalis* (GardV, red fluorescence) is present within this mix but is not adherent to the epithelium. Similarly composed groups of bacteria can be found attached to the torn site of the same biopsy specimen. The picture on the right **(B)** demonstrates hybridization with the universal probe at magnification of x400 and a triple contrasted inset at magnification of x1,000. Bacteria attached to the injured surface originate from vaginal secretions and are not primary residents of the epithelium.

Swidsinski. Biofilms in Bacterial Vaginosis. Obstet Gynecol 2005.

woman. However, the proportion of the Lab-positive (*Lactobacillus*) bacteria reached between 1% and 5% in only 20% of the biopsy specimens with adherent biofilm. In all other patients with adherent biofilm, Lab-positive (*Lactobacillus*) bacteria were found in concentrations of less than 10^6, thus contributing less than 0.01% to the biofilm.

The proportion of Cor-positive (*Coriobacterium*) bacteria reached 15% in 1 woman with bacterial vaginosis and adherent *G vaginalis* biofilm. Bacteria that positively hybridized with the Cor (*Coriobacterium*) probe were absent or present in low concentrations of less than 10^6 CFU/mL in all other patients and controls. Bacteria that hybridized with the probes Bac (*Bacteroides*), Veil (*Veillonella*), Strc (*Streptococcus*), Rbro (*Ruminococcus*), Ehal (*Eubacterium hallii*), and Arc (*Arcobacter*) were found in a small set of biopsy specimens (Table 3); however, none of these groups contributed more than 0.1% to the biofilm.

Adherent bacteria were not distinguishable from the eukaryotic structures using hematoxylin-eosin, methenamine silver, or standard Gram stains, despite the high concentrations of bacteria present within adherent biofilms. However, the biofilm was readily apparent using the Brown-Hopps modification of the Gram stain (Fig. 4). Electron microscopy further confirmed the presence of bacteria adherent to the mucosal surface of these biopsy specimens (Fig. 5).

Bacteria hybridizing with probes that recognized the HGC (*Actinobacteria*) cluster were most predominant in samples of vaginal biopsies from women with bacterial vaginosis. These bacteria hybridized with the HGC (*Actinobacteria*) probe and included subgroups that also positively hybridized with Bif164, Bif662 (*Bifidobacteriaceae*), GardV (*Gardnerella*), Ato (*Atopobium*), or Cor (*Coriobacterium*) probes. Hybridization with the Bif1278 (*Bifidobacterium*) probe was negative in all subjects.

The relevance of single fluorescence in situ hybridization probes for the characterization of the biofilm constituents differed. The Bif164, Bif662 (*Bifidobacteriaceae*), and GardV (*Gardnerella*) probes hybridized with the same bacteria within the biofilm. These bacteria were responsible for 70% to 99% of the mass of the adherent biofilms found in women with bacterial vaginosis but were only found as single scattered organisms within the unstructured biofilms of the control groups. The GardV Cy-5 probe (red fluorescence, Fig. 3) that contained no mismatches with *G vaginalis* provided the most brilliant pictures and the best separation between background fluorescence and bacterial fluorescence, allowing the quantification of nearly each bacterial cell. The fluorescence of Bif164 and Bif662 Cy-5 probes (*Bifidobacteriaceae*, 1 mismatch with *G vaginalis*), was less brilliant but still sufficient for identification and

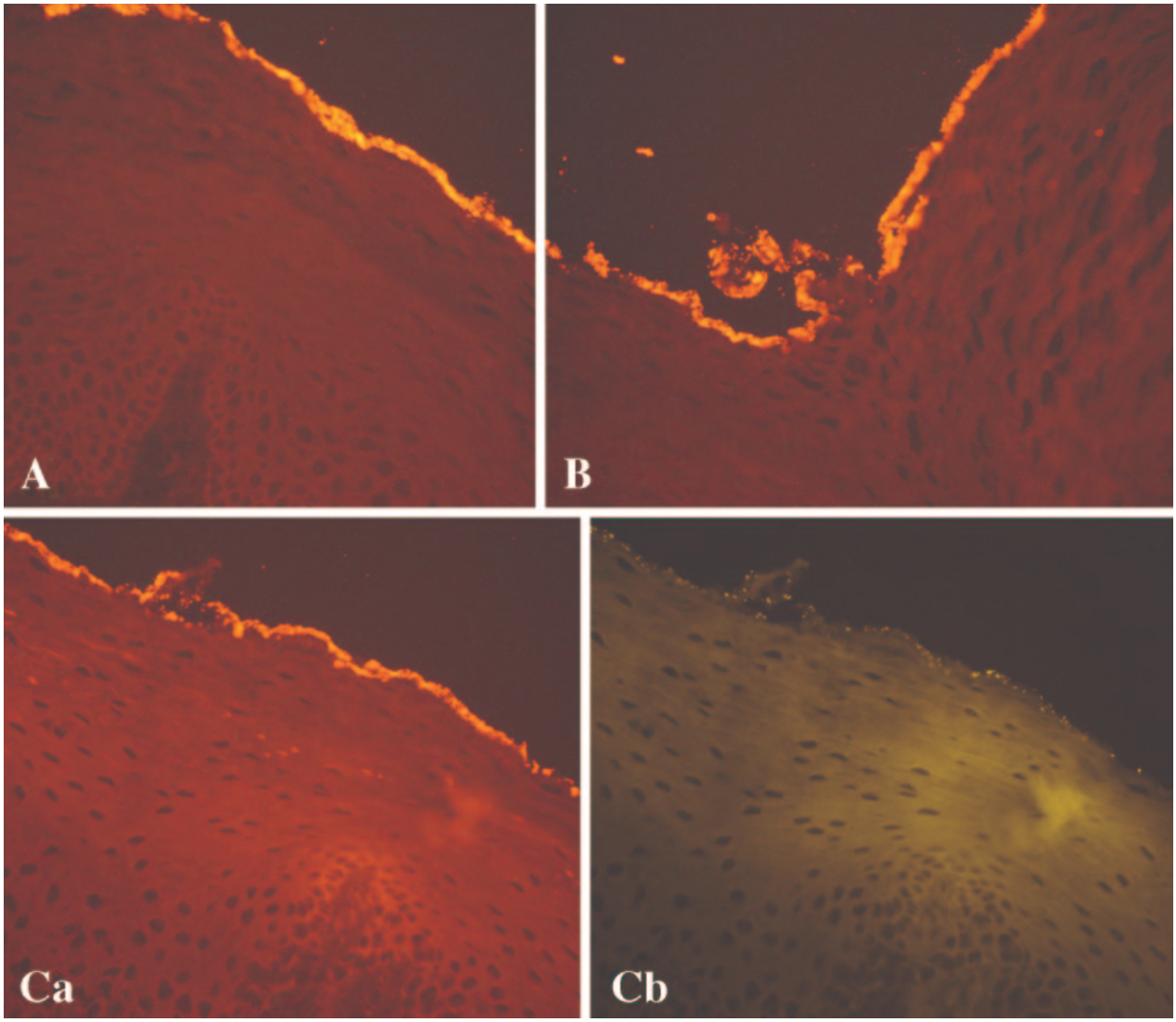

Fig. 3. Three adjacent microscopic fields **(A, B, C)** show a continuous *Gardnerella vaginalis* biofilm covering the vaginal epithelium (visualized with the GardV-Cy5 probe, red fluorescence, x400). The 2 pictures below show the same microscopic field separately in dark red fluorescence (**Ca**, GardV-Cy5 probe, bottom left) and orange fluorescence (**Cb**, Lab Cy-3, bottom right). Bacteria that positively hybridized with the Lab (*Lactobacillus*) probe are present but are heterogeneously woven into the *G vaginalis* biofilm and contribute only to 5% of the whole bacterial population.

Swidsinski. Biofilms in Bacterial Vaginosis. Obstet Gynecol 2005.

enumeration of *G vaginalis*. Eighty-six percent of the adherent GardV (*Gardnerella*) biofilms were further associated with high concentrations of Ato-positive (*Atopobium*) bacteria, which could reach up to 40% of the biofilm mass or concentrations of 4×10^{10} bacteria per millimeter. Although Ato-positive bacteria were also detected in the control groups with unstructured biofilm, their concentration in these patients was less than 1% of the population or 10^5 bacteria per millimeter. Cor-positive (*Coriobacterium*) bacteria contributed to 15% of the adherent biofilm in 1 woman with bacterial vaginosis. In all other subjects, only single Cor-positive bacteria were seen (Table 3). Representatives of the LGC (*Firmicutes*) cluster were found in almost all biopsy specimens, including bacteria that also positively hybridized with the Lab (*Lactobacillus*), Str (*Streptococcus*), and Enc (*Enterococcus*) probes. LGC/Lab-positive bacteria were found in all but 1 woman with bacterial vaginosis. The concentrations of Lab-positive (*Lactobacillus*) bacteria were low in most healthy controls and 60% of subjects with bacterial vaginosis, but could reach considerable

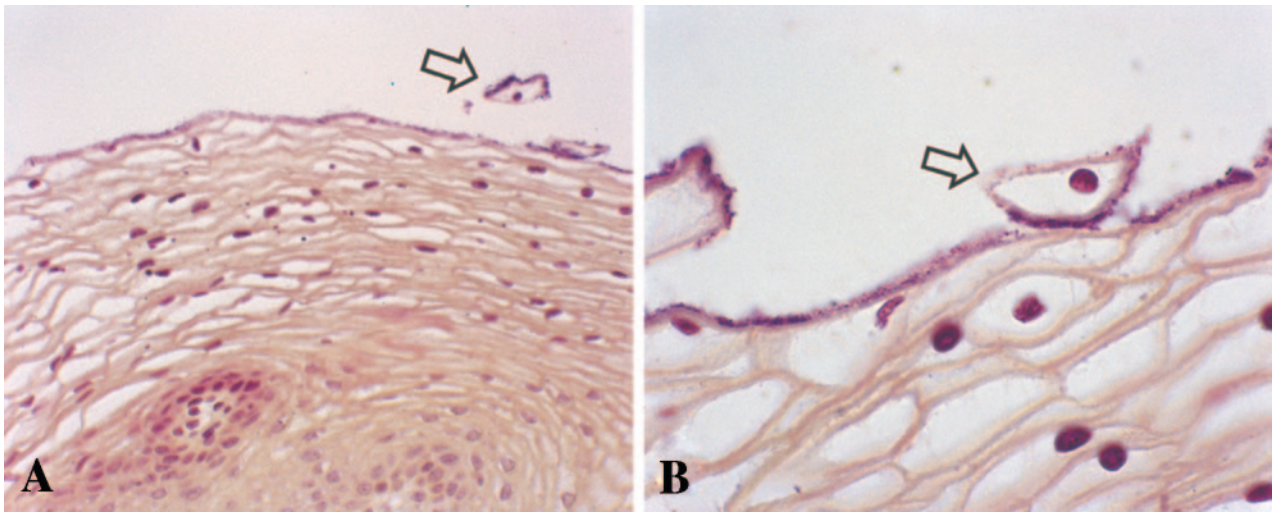

Fig. 4. A continuous biofilm can be detected histologically on the vaginal epithelial surface in patients with bacterial vaginosis (Brown-Hopps modification of the Gram stain). Original magnifications: left panel, x100 **(A)**; right panel, x250 **(B)**. Note the desquamation of surface epithelial cells containing the biofilm that can be detected as ''clue cells'' in the vaginal smear *(arrows)*.

Swidsinski. Biofilms in Bacterial Vaginosis. Obstet Gynecol 2005.

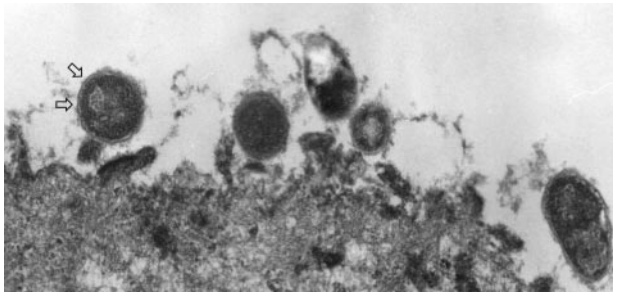

Fig. 5. Electron microscopy confirms the presence of gram-positive bacteria adherent to the surface of the vaginal epithelium. Note the characteristic cell wall *(arrows)*.

Swidsinski. Biofilms in Bacterial Vaginosis. Obstet Gynecol 2005.

numbers of up to 1.3×10^{10}/mL and formed unstructured biofilms. The unstructured Lab-predominant (*Lactobacillus*) biofilms were especially common in postmenopausal women (55%). These biofilms were composed of thick long rods of different length that were suspended in secretions overlying the mucosal surface.

The proportion of all other groups of bacteria in the biofilm was low. Bacteria hybridizing with the Strc (*Streptococcus*), Ato (*Atopobium*), Arc (*Arcobacter*), Bac (*Bacteroides*), Veil (*Veillonella*), Rbro (*Ruminococcus*), and Clit (*Clostridium lituseburense*) probes were occasionally found within both adherent and unstructured biofilms. However, none of these subpopulations reached a threshold of 1% or concentrations higher than 10^5 bacteria per millimeter. The Bif1278 (*Bifidobacterium*), Ebac (*Enterobacteriaceae*), Erec (*Eubacterium rectale*), Phasco (*Phascolarctobacterium*), Chis (*Clostridium histolyticum*), Ser (*Brachyspira*), Lach (*Lachnospira*), Bdis (*Bacteroides distasonis*), Fprau (*Fusobacterium prausnitzii*), CF (*Cytophaga-Flavobacteria*), Y16s-69 (*Yersinia*), Sfb (*Segmented filamentous bacteria*), Hpy-1 (*Helicobacter pylori*), and Aer (*Aeromonas*) probes failed to give signals different from the background fluorescence seen with the nonsense probe.

The GardV-positive (*Gardnerella*) biofilm was found in 18 of 20 women with bacterial vaginosis and in 1 of 20 premenopausal and 3 of 20 postmenopausal controls. Repeat investigation of the 4 women with adherent GardV+ Biofilm, who were initially subjects in the control groups for lack of symptoms and normal appearance during their clinical investigation including the Gram stain investigation of smears, showed that these 4 subjects actually had a moderate form of bacterial vaginosis. Two women in the bacterial vaginosis group without a GardV+ biofilm had vaginal cultures with massive overgrowth of *Candida albicans* and absence of *G vaginalis*. Thus it is likely that these patients had candidiasis that was misdiagnosed as bacterial vaginosis.

Each of the *G vaginalis* strains isolated from patients in this study positively hybridized with the Bif164, Bif662 (*Bifidobacteriaceae*), GardV (*Gardnerella*), and HGC (*Actinobacteria*) probes but not the Bif1278 (*Bifidobacterium*) probe. Other isolated bacteria including *Corynebacteria* and *Mobiluncus* did not hybridize with the Bif164, Bif662, GardV, or HGC probes. No *Bifidobacteria* were isolated from vaginal swabs.

DISCUSSION

Bacterial biofilms are increasingly identified as a source of many recalcitrant bacterial infections such as periodontal disease, endocarditis, foreign body–related infections. They are also an important pathogenetic factor in chronic conditions such as obstructive lung disease, inflammatory bowel disease, and gallstone formation.[9,33,34] Based on fluorescence in situ hybridization of vaginal biopsy specimens, we found that bacterial vaginosis is associated with the development of an adherent polymicrobial biofilm containing abundant GardV+ bacteria (*Gardnerella*) on the vaginal epithelium. In agreement with previous studies, *G vaginalis* is predominant in vaginal cultures from subjects with bacterial vaginosis, and *Lactobacilli* are predominant in the vagina of healthy premenopausal women. The diagnosis of bacterial vaginosis correlated with more frequent detection of other bacterial groups such as *Atopobium, Bacteroides, and Veillonella* by vaginal cultures, as previously reported.[2,7,8]

The biofilm found in bacterial vaginosis is highly organized. Short, nearly coccoid rods that positively hybridized with the GardV (*Gardnerella*) probe were stacked in confluent or patchy layers that were tightly attached to the surface of the vaginal epithelium. GardV+ bacteria were a predominant and obligatory component of the biofilm, reaching concentrations up to 10^{11} bacteria per millimeter. However, other types of bacteria were also present within the biofilms. Bacteria that positively hybridized with the Ato probe (*Atopobium*) were found in 80% of the GardV-positive (*Gardnerella*) biofilms and contributed up to 40% of the biofilm mass. The occurrence of other bacterial groups within the GardV-predominant biofilm was markedly higher than seen in the control groups without biofilms, providing an explanation for the increased quantity and diversity of multiple bacterial groups cultured from patients with bacterial vaginosis. However, with exception of the Ato-positive (*Atopobium*) bacteria, all other bacterial groups detected within the biofilm were irregularly intermixed and showed no specific patterns of occurrence or spatial organization.

Gardner and Dukes[2] recognized the value of vaginal clue cells for the diagnosis of bacterial vaginosis. Clue cells are squamous epithelial cells whose surfaces are heavily coated with bacteria. Our data show that squamous epithelial cells become coated with bacteria (the biofilm) in situ within the mucosa (Fig. 4). Subsequent desquamation of these cells results in formation of classic clue cells.

In contrast to bacterial vaginosis, adherent biofilms were not observed on the epithelium of most healthy controls. Unstructured accumulations of bacteria within secretions (90% long rods that positively hybridized with the *Lactobacillus* probe) were loosely attached to specimen surfaces in 25% of premenopausal and 55% of postmenopausal women. These loose bacterial accumulations were found both on the epithelial and subepithelial surfaces of the biopsy specimens, suggesting that they derived mainly from vaginal secretions that envelop traumatized biopsy specimens and were not primary adherent.

Many of the probes used in this study failed to give signals that could be distinguished from background autofluorescence of the nonsense Eub probe (negative control for bacteria). Thus, no Ebac-positive (*Enterobacteriaceae*) bacteria were detected, even in women who had positive *Escherichia coli* cultures with concentrations of 10^{3-4} CFU/mL. Bacteria that hybridized with probes representing big *Clostridia* groups were found extremely infrequently and did not correlate with clostridia cultured from vaginal smears. However, with bacterial concentrations of 10^5 or less per milliliter, a maximum of 1 cell would be found in 20 consecutive microscopic fields. Because most biopsy specimens contained 12–20 fields showing intact vaginal surface, concentrations lower than 10^4 bacteria per milliliter simply could not be detected by fluorescence in situ hybridization. These low concentrations were, however, numerically irrelevant for biofilm detection.

Our data on the composition of vaginal flora are incomplete due to these reasons and lack of available group-specific fluorescence in situ hybridization probes for all potentially relevant bacterial groups. For example we still have no reliable fluorescence in situ hybridization probes specific for *Mobiluncus* or *Mycoplasma.* However, it is only a question of time until those probes are developed and the gaps are closed using a combination of different microbiologic methods.

In summary, fluorescence in situ hybridization evaluation of vaginal biopsy specimens demonstrates that the presence of an adherent vaginal biofilm that predominately hybridizes with a *G vaginalis* probe is sensitive and specific for detecting bacterial vaginosis. Although more studies are needed, these observations raise the possibility that the development of this biofilm may be responsible for the pathogenesis of bacterial vaginosis, rather than gradual overgrowth of resident vaginal flora within vaginal secretions as hypothesized previously. Previous electron microscope studies described an electron-dense web-like fibrillar exopolysaccharide connecting *G vaginalis* cells to each other and to the epithelial cells.[35,36] However, the requirement of biofilm formation for the development of bacterial vagi-

nosis was not recognized, and the question of whether the *G vaginalis* strains and other bacteria isolated from bacterial vaginosis patients and healthy controls are the same entities with regard to their adhesive properties was not pursued. The present study may allow other investigators to focus on the epithelial cell surface in bacterial vaginosis.

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