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Gardnerella Biofilm Involves Females and Males and Is Transmitted Sexually

Alexander Swidsinski[a] Yvonne Doerffel[b] Vera Loening-Baucke[a]
Sonja Swidsinski[e] Hans Verstraelen[f] Mario Vaneechoutte[g] Vesna Lemm[d]
Johannes Schilling[a] Werner Mendling[c]

[a]Laboratory for Molecular Genetics, Polymicrobial Infections and Bacterial Biofilms, Charité, Gastroenterology, and [b]Outpatient Clinic of the Charité, CCM, Humboldt University, [c]Clinics for Gynecology and Obstetrics, Vivantes Hospital, Klinikum am Urban and im Friedrichshain, [d]General Practice for Obstetrics and Gynecology, and [e]Department of Microbiology, Vivantes Hospital, Neukölln, Berlin, Germany; [f]Department of Obstetrics and Gynaecology, Ghent University, Ghent University Hospital, and [g]Laboratory of Bacteriology Research, Department of Clinical Chemistry, Microbiology and Immunology, University Hospital, Ghent, Belgium

Key Words
Gardnerella biofilm · Bacterial vaginosis · Fluorescence in situ hybridization

Abstract
Objective: To study the incidence and distribution of adherent *Gardnerella vaginalis*. ***Methods:*** Bacteria adherent to desquamated epithelial cells in the urine were detected using fluorescence in situ hybridization (FISH). Urine from patients with bacterial vaginosis (BV, n = 20), their partners (n = 10) and different control populations (n = 344) including pregnant women and their partners, randomly selected populations of hospitalized man, women and children as also healthy controls was investigated. ***Results:*** *Gardnerella* was found in two different forms: cohesive and dispersed. In the cohesive form, *Gardnerella* were attached to the epithelial cells in groups of highly concentrated bacteria. In the dispersed form, solitary *Gardnerella* were intermixed with other bacterial groups. Cohesive *Gardnerella* was present in all patients with proven BV and their partners, in 7% of men and 13% of women hospitalized for reasons other than BV, in 16% of pregnant women and 12% of their male partners, and in none of the healthy laboratory staff or children. In sexual partners, occurrence of cohesive *Gardnerella* was clearly linked. Dispersed *Gardnerella* were found in 10–18% of randomly selected females, 3–4% of males and 10% of children and not sexually linked. In daily longitudinal investigations over 4 weeks no transition between cohesive and dispersed *Gardnerella* and vice versa was observed. Transmission of a cohesive *Gardnerella* strain could be followed retrospectively over 15 years using molecular genetic methods. ***Conclusions:*** Cohesive *Gardnerella* biofilm is a distinct, clearly definable entity which involves both genders and is sexually transmitted. The correct name distinguishing it from symptom-defined conditions like BV should be gardnerellosis and for the bacterium *Gardnerella genitalis*.

Introduction

Bacterial vaginosis (BV) is an old and still unsolved infectious riddle [1]. Until now, neither the pathogen nor the exact requirements for development of BV were apparent [2–6]. We have previously analyzed the bacterial

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Dr. Alexander Swidsinski
Charité
Hessische Strasse 3–4
DE–10098 Berlin (Germany)
Tel. +49 30 450 514 003, E-Mail alexander.swidsinski@charite.de

microbiota in vaginal biopsy material using fluorescence in situ hybridization (FISH) and demonstrated that BV is associated with a prolific polymicrobial biofilm [3]. *Gardnerella vaginalis* was the obligate and the predominant component of the BV biofilm. The epithelial cells desquamating from the vaginal epithelium were primarily (in situ) enclosed in this bacterial coat, leading to the formation of clue cells. In contrast, the epithelium of vaginal biopsies from healthy controls had no adherent bacteria and the surface of desquamating epithelial cells was clean.

The present study investigates the occurrence of biofilm-forming *Gardnerella* in urine samples of different cohorts horizontally (women with typical BV; randomly selected populations of female, male, and pediatric patients treated in a general hospital for reasons other than BV), in pairs (pregnant women and their sexual partners), and longitudinally (healthy male and female volunteers and BV patients who provided daily urine sample over a 4-week period).

Vaginal epithelial cells are also present in smears of vaginal swabs. The FISH analysis of the bacterial community requires multiple hybridizations, which should be carried out under exactly reproducible conditions, a premise that cannot be achieved when using smears from vaginal swabs because smears vary strongly in thickness and composition of cell debris and amounts of dried vaginal secretions. Since many vaginal epithelial cells are washed down while spontaneously voiding urine, bacteria adherent to them can be investigated noninvasively, replacing the vaginal biopsy investigations. We chose urine samples for practical reasons after comparative pilot investigations. Both sessile, attached to the epithelial surface, and bacteria suspended in urine can be investigated. Urine sediments fixated in Carnoy solution [3] can be stored for long periods of time and the aliquots can be used for repeated hybridizations under standardized conditions. The urine samples can be delivered daily, without the need for a physician, allowing the longitudinal monitoring of the findings. Our aim was to study the incidence and distribution of adherent *G. vaginalis* in urine using FISH.

Methods

Patients and Material

(1) Twenty women from a general gynecologic practice with symptomatic BV, diagnosed according to the Amsel criteria, and 10 of their sexual partners were evaluated. From each of these women, a vaginal biopsy, vaginal smear and urine sample were obtained. Sections of the biopsies were investigated using FISH as previously described [3]. Vaginal smears were Gram stained and interpreted according to Nugent. All of the women had a Nugent score of more than 6 and all had an adherent bacterial biofilm covering the epithelial surface.

Two women with BV supplied fresh urine samples daily, on weekdays only, for a 4- to 8-week period.

(2) 100 females and 100 males (>18 years of age) and 50 girls (4–10 years of age), who were hospitalized for reasons other than BV on the surgical, internal medicine or pediatric wards and who had urine samples sent for culture to the microbiologic laboratory were studied. An aliquot of urine was obtained for FISH investigation.

(3) Urine samples were obtained for FISH investigation from 72 married pregnant women visiting a general physician for routine screening prior to childbirth, and from all of their partners. Additionally, vaginal smears were performed on each woman and interpreted using the Nugent score. None of the male partners was circumcised.

(4) A total of 20 healthy volunteers (10 females, 5 uncircumcised males and 5 children) recruited from laboratory personnel and 2 outpatients with bacterial vaginosis supplied fresh urine samples daily, on weekdays only, for a 4-week period.

Of note is that circumcision is rarely performed in Germany.

Sample Collection

A 2-ml urine aliquot was collected from all urine samples from hospitalized patients whose urine was sent to the bacteriologic laboratory. The urine samples from outpatients and volunteers were directly delivered to the Molecular Genetic Laboratory for Polymicrobial Infections and Bacterial Pathogens at the Charité Hospital, Berlin, Germany.

Handling of Urine Samples

The urine samples were fixated in Carnoy solution (6/6/1 vol ethanol/glacial acetic acid/chloroform) on the same day. An aliquot of 1.5 ml urine was centrifuged in a 1.5-ml Eppendorf tube for 6 min at 6,000 *g*. The sediment was decanted, the tube was filled with 1 ml of Carnoy solution and left at room temperature. After 60 min, the sediment was centrifuged once more (6 min/6,000 *g*), decanted, 75 μl Carnoy solution was added, and then the sample was stored at 4°C. The initial FISH analysis for this study was performed within one week after fixation of the urine sample. Some of the investigations were repeated up to 4 months later and gave the same results. The 5 × 5-mm quadrant area of hybridization was marked with a PAP Pen on a superfrost glass slide. The Carnoy-fixated urine sediment was vortexed, 5-μl aliquots (representing 100 μl of the initial urine volume) were pipetted within the area of hybridization and dried for 30 min at 50°C just prior to hybridization. The sediments on the glass slides were incubated with 20 μl of 1% lysozyme for 15 min at room temperature prior to hybridization.

Concentrations of epithelial cells in the urine sediments were calculated per ml urine. The numbers of adherent bacteria were enumerated per epithelial cell (maximal and mean per sample). The concentrations of adherent bacteria in the urine were calculated by multiplying the mean number per epithelial cell with the concentration of epithelial cells per milliliter.

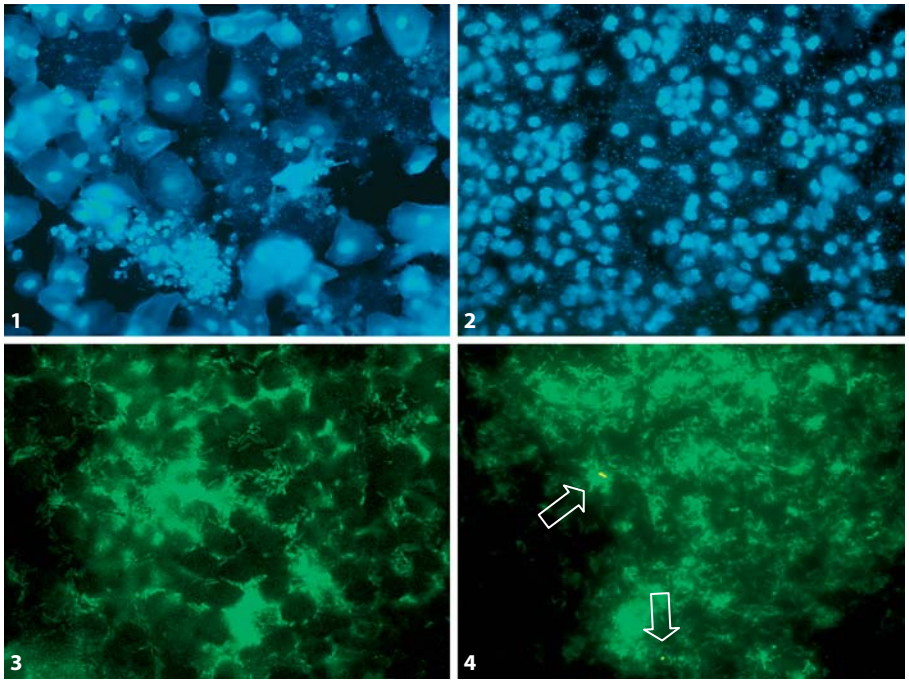

Fig. 1. DAPI stain of female urine sediment shows accumulations of desquamated epithelial cells (large polygonal cells) and groups of leukocytes with smaller nuclei and cells. ×400.

Fig. 2. Male urine sediment with massive leukocytes and multiple fungi located between leukocytes. ×400.

Fig. 3. Diffusely distributed (nonattached) bacteria with negative outline (holes) of epithelial cells within a homogeneous carpet of bacteria (universal probe Eub338-FITC, green signals). ×400.

Fig. 4. Single cells of *Gardnerella* (dispersed *Gardnerella,* Bif164-Cy3 probe, yellow signals, arrows, the bacteria were also positive with GardV-Cy5 probe, signals not shown) within an accumulation of other bacterial groups (Eub338-FITC). ×400.

FISH

We used a Nikon e600 fluorescence microscope, Nikon DXM1200 camera and accompanying software (Nikon, Tokyo, Japan).

Bacteria were assessed in a multicolor analysis using a mix of three probes: Bif164-Cy3/Eub338-FITC/GardV-Cy5 [3] and DAPI counterstain [3].

Genotyping

G. vaginalis isolates were genotyped using alkaline DNA extraction and RAPD analysis using the RAPD Ready-to-Go beads (GE Healthcare, Buckinghamshire, UK) with primer OPM1 (5′ GTT GGT GGC T) [7]. Reactions were carried out in a 12-μl volume containing 10.8 μl of a PCR mix. A volume of 1.2 μl of sample DNA was added per tube. After 5 min at 94°C, reaction mixtures were cycled 30 times with the following conditions: 30 s at 94°C, 1 min at 35°C, and 1 min at 72°C, with a final extension period of 5 min at 72°C. Reaction vials were then cooled to 10°C and kept on ice until used in electrophoresis.

Capillary Electrophoresis

12 μl of deionized formamide was mixed with 0.5 μl of an internal size standard mixture containing 0.3 μl of the GS-400 high-density size standard and 0.2 ml of the GS-500 size standard (Applied Biosystems, Foster City, Calif., USA), which both contain ROX-labeled fragments in the range of 50–500 bp. 1 μl of OPM1-PCR product was added. The mixtures were denatured by heating at 95°C for 3 min and placed directly on ice for at least 15 min (according to the recommendations of the manufacturer).

Capillary electrophoresis was carried out using an ABI-Prism 310 genetic analyzer (Applied Biosystems) at 60°C, at a constant voltage of 1.5 kV, and at a more or less constant current of approximately 10 mA. Capillaries with a length of 47 cm and diameter of 50 mm were filled with performance-optimized polymer 4. Electropherograms were normalized using Genescan Analysis software, version 2.1 (Applied Biosystems) and further analysis was carried out with BaseHopper [8].

Results

Microscopy of Urine Sediments

Urine sediment included bacteria, epithelial cells, leukocytes, spermatocytes, fungi and polymorph composed cell debris (fig. 1–4). The differentiation of eukaryotic cells and bacteria in urine sediments was easy in hematoxylin and eosin, PAS and many other classical histologic stains. Light microscopy could not be simultaneously performed with FISH. However, all of the mentioned cell types could be recognized in DAPI stain. The desquamated epithelial cells were 3–10 times bigger than leukocytes, had irregular polygonal forms and a markedly broader and optically dense cytoplasm surrounding the epithelial cell nucleus (fig. 1). The leukocytes were round, nearly completely filled with a fluorescent nucleus and with a narrow cytoplasm which was practically not apparent in DAPI (fig. 1, 2). The fungi were smaller than epithelial cells and leukocytes and unmistakably different to other eukaryotic structures (fig. 2).

Spatial Distribution of Bacteria in Urine Sediments

Routine microbial culture analysis of urine samples was available for all hospitalized patients. Generally, in urine samples with bacteriuria of more than 10^6 colony-forming units (cfu) per milliliter, bacteria were found diffusely distributed over the glass surface representing a diffusely suspended bacterial population. The epithelial cells appeared as holes within a dense microbial carpet (fig. 3), indicating that the bacteria were not truly attached to the epithelial cells. Theoretically, FISH of urine samples with bacteriuria of $<10^3$ cfu/ml should show at most 4 bacteria per microscopic field at a magnification of ×100. This was not true in cases of sessile or adherent bacteria. The numbers of bacteria attached to desquamated epithelial cells could exceed 400. When plated, such conglomerates of bacteria will give rise to a single colony.

The adherence to epithelial cells was typical for *Gardnerella* in BV (fig. 5) but also for other bacterial groups occurring in high numbers in the subset of healthy women (no picture shown). Unlike the negative outlines of epithelial cells observed in patients with bacteriuria (fig. 3), adherent bacteria highlighted the outlines of the epithelial cells (fig. 4). The spatial relation of bacteria to desquamated epithelial cells allowed us to divide urogenital bacteria in urinary tract-associated bacteria (UTAB) and genital epithelia-associated bacteria (GEAB). We are exclusively referring to genital epithelia-associated bacteria in the remainder of this work.

Pitfalls of the Bacterial Evaluation

A low number of epithelial cells in the urine automatically lead to a low concentration of genital epithelia-associated bacteria. With epithelial cell numbers of less than 5 per whole area of microscopic evaluation (5 × 5 mm), the detection of GEAB bacteria was accidental. To compensate for the low concentration of epithelial cells in such samples, we centrifuged the sediments once more and repeated the hybridizations with the 10 times concentrated urine samples. If the number of epithelial cells was still lower than 5, the genital bacteria were regarded as nonanalyzable.

Complex materials such as histological sections or urine sediments contain different components, which can nonspecifically bind fluorochromes and generate signals which are difficult to distinguish from true hybridization signals. We found in preliminary investigations that signals mimicking single GardV-positive bacteria could be occasionally seen within eukaryotic debris or accumulations of bacteria other than *Gardnerella*. We applied a mix of three probes that hybridized with different regions of the *Gardnerella* species (Bif164-Cy3/Eub338-FITC/GardV-Cy5) to ensure the specificity of the findings. Only signals which were simultaneously positive for *Bifidobacteriaceae* (Bif164-Cy3 probe), *Gardnerella* (GardV-Cy5 probe) and universal for all bacteria (Eub338-FITC probe) and had a counterpart in the DAPI stain were regarded as positive. We had no difficulties to differentiate between true hybridization signals and biases using this set of probes.

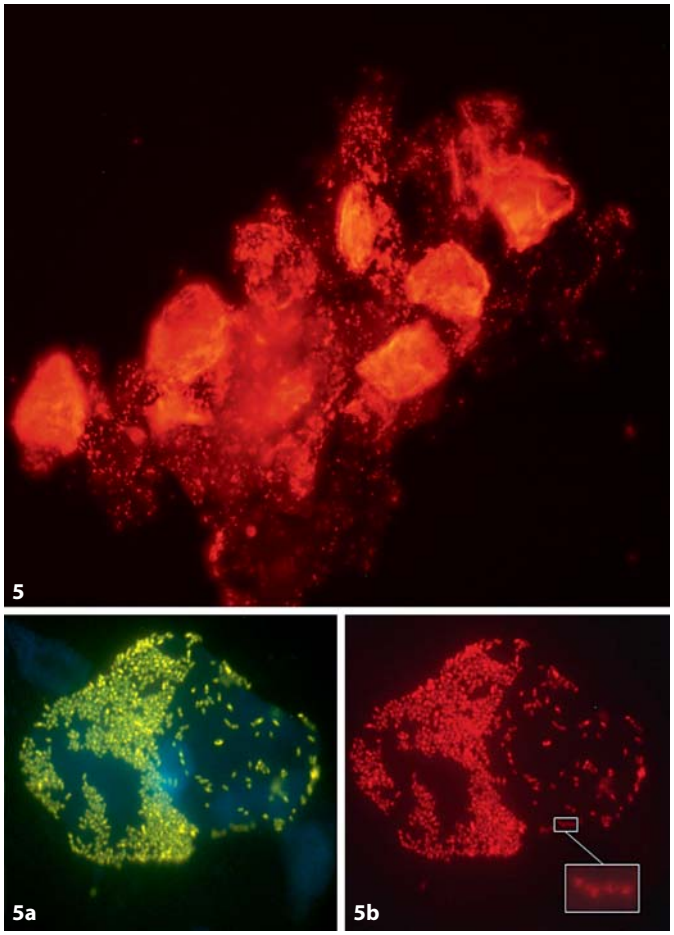

Fig. 5. *Gardnerella* (GardV-Cy5 probe, red fluorescence)-coated cells in patient with BV. ×400. Peculiarity of cohesive *Gardnerella* arrangement to structures resembling brickwork are demonstrated: **a** Bif164-Cy3, *Bifidobacteriaceae*, yellow fluorescence, ×1,000; **b** GardV-Cy5, *Gardnerella*, red fluorescence, same microscopic field. *Gardnerella* is a short rod with a dark spot in the center of the body. Because of this spot, the bacterium can be mistaken as a short chain of cocci (inset fig. 5b).

Shape, Appearance, and Spatial Arrangement of Gardnerella *Bacteria*

Bif164/GardV-positive bacteria occurred in two markedly different forms: dispersed and cohesive *Gardnerella* (fig. 4, 5). The dispersed *Gardnerella* was always a marginal component of the vaginal microbiota which hybridized with the universal bacterial probe (Eub338; fig. 4). The dispersed *Gardnerella* could be sometimes concentrated to small groups of 10–20 bacteria surrounding leukocytes but not contacting epithelial cells. However, even within these accumulations of bacteria, single Bif164/GardV-positive bacteria were distinctly separated from each other and we never observed more than 5 *Gardnerella* bacteria that were joined together. In contrast, cohesive *Gardnerella* cells were always numerically significant within the GEAB population (fig. 5) and made up at least 5% to more than 90% of the Eub338-positive bacteria. The highest concentrations of cohesive *Gardnerella* were observed on the surface of epithelial cells. Up to a thousand bacteria were attached to a single epithelial cell and were considered remnants of the *Gardnerella* biofilm. Cohesive *Gardnerella* were arranged as a structure similar in appearance to brickwork in regions of high density (fig. 5a, b).

The distribution of cohesive *Gardnerella* could differ between different regions of the same urine sediment especially when only small numbers of epithelial cells were present. The urine sample was regarded as positive for cohesive *Gardnerella* if even one epithelial cell was covered with cohesive *Gardnerella*, and the presence of single scattered *Gardnerella* in other regions, which in the absence of cohesive *Gardnerella* would be regarded as dispersed *Gardnerella*, was ignored.

Both cohesive and dispersed *Gardnerella* had the shape of short rods. The rods of *Gardnerella* often remained interconnected at one of the poles after cell division and thus appeared to be longer than they actually were. The rods had a dark spot in the center. Because of this dark spot, the pair of short rods with their round dark centers could be mistaken for a short chain of four cocci during superficial inspection (fig. 5b, inset) and the accumulations of cohesive *Gardnerella* rods could be mistaken for a mat of cocci. The morphology of dispersed *Gardnerella* was unmistakable, since bacteria were generally larger and longer and never clenched.

Occurrence of Gardnerella *in Different Control Groups* (table 1)

Randomly Selected Hospitalized Patients (n = 250)

The occurrence of cohesive *Gardnerella* in the unselected hospitalized population was 13% in the females and 7% in the males. No cohesive *Gardnerella* were identified in the urine samples of girls (n = 50). The concentrations of cohesive *Gardnerella* in unselected hospitalized patients were lower than in patients with BV and their partners.

The occurrence rate of dispersed *Gardnerella* was 22% in females, 4% in males and 10% in girls.

Longitudinal Investigations of Urine Samples in Diseased and Healthy Volunteers

Impact of the Prepuce on the Number of Epithelial Cells in Urine Samples. Urine sediments from unselected hospitalized women contained 10–10,000 desquamated epithelial cells per area of microscopic evaluation (approximately 25 microscopic fields at magnification ×100). The variation was similar in men (0–10,000 epithelial cells). However, 18% of the male and none of the female urine samples contained less than 5 visible cells.

Three of the healthy male volunteers delivered two urine samples per day for 10 days. One sample had to be obtained with the prepuce left over the glans penis, the other with the prepuce pulled back. The difference was marked. When the prepuce was pulled back, the number of epithelial cells was low and 65% of the samples were nonanalyzable. When the prepuce was covering the glans penis, the number of epithelial cells was significantly higher and only one urine sample was nonanalyzable (table 1).

We therefore recommended male probands to leave the prepuce over the glans penis for sample collection. We also recommended to both males and females to avoid cleaning the genitals before sample collection. Since the urine sample collection from unselected hospitalized patients was performed without special instruction, we did not compare the differences in epithelial cell number between hospitalized patients and controls.

Longitudinal Changes of Cohesive Gardnerella *in Bacterial Vaginosis.* Two of the BV patients delivered urine samples daily for 4 weeks. All of these samples were positive for cohesive *Gardnerella*; however, the proportion of cohesive *Gardnerella* to the total number of bacteria varied between 40 and 90% from week to week.

Longitudinal Changes in 20 Healthy Controls

Cohesive *Gardnerella* were absent in all samples from healthy male, female and girls (table 1). Dispersed *Gardnerella* were detected in 25% of longitudinally taken urine samples in 2 healthy female volunteers (4 and 9 samples each), in 3 samples from 1 male volunteer and in 5 samples from 1 of the child volunteers. In each of the indi-

Table 1. Occurrence of *Gardnerella* in different patient groups and control groups

Number of patients/samples	Concentration of epithelial cells/ml $\times 10^4$ (mean ± SD)	Cohesive *Gardnerella*			Dispersed *Gardnerella*			Non-analyzable samples %
		occurrence of cohesive *Gardnerella* (patients/samples), %	max./average number of bacteria per epithelial cell (mean ± SD)	concentrations per ml (10^6 bacteria/ml)	occurrence of dispersed *Gardnerella* (patients/samples), %	max./average number per epithelial cell	concentrations per ml (10^6 bacteria/ml)	
BV (20/20)	2.7 ± 2.9	100	260 ± 112/54 ± 49	2.3 ± 4.7				0
BV partner (10/10)	0.6 ± 1.09	100	129 ± 67/43 ± 30	0.25 ± 0.49				0
Unselected hospitalized patients								
Female (100/100)	0.9 ± 1.9	13	180 ± 140/24 ± 31	0.32 ± 0.7	22	4 ± 4/0.08 ± 0.06	0.0006 ± 0.0006	0
Male (100/100)	0.2 ± 0.4	7	89 ± 54/34 ± 32	0.17 ± 0.37	4	3 ± 1.4/0.08 ± 0.04	0.00008 ± 0.00007	18
Children (50/50)	0.2 ± 0.5	0	–	–	10	3 ± 1.6/0.05 ± 0.05	0.0004 ± 0.00006	7
Partners awaiting childbirth								
Pregnant women (72/144)	0.5 ± 0.9	17/12	200 ± 202/18 ± 21	0.5 ± 1.0	14/8	5 ± 5/0.10 ± 0.08	0.0006 ± 0.0006	3
Partners of pregnant women (72/144)	0.1 ± 0.2	11/6	68 ± 54/34 ± 32	0.14 ± 0.31	3/0.5	1–2	0.00005 ± 0.00007	12
Longitudinal samples from healthy volunteers								
BV 1 (1/20)	3.1 ± 2.9	100	190 ± 136/39 ± 37	3.1 ± 3.7				
BV 2 (1/42)	1.0 ± 0.8	100	76 ± 44/18 ± 16	1.3 ± 2.1				
Healthy women (10/200)	1.2 ± 1.8	0			20/7	2.2 ± 1.1/0.08 ± 0.03	0.0009 ± 0.0008	0
Healthy children (5/100)	0.2 ± 0.4	0			10/5	4.1 ± 1.6/0.1 ± 0.06	0.0003 ± 0.0004	9
Healthy men with glans penis exposed (3/30)	0.0008 ± 0.02	0			0	0		65
Healthy men with glans penis covered (5/100)	0.5 ± 0.81	0			1/3	1–4		1

viduals, the presence of dispersed *Gardnerella* was not concurrent but irregularly alternated with the absence of *Gardnerella*, and the concentrations of the dispersed *Gardnerella* were low. In none of the samples could dispersed *Gardnerella* be mistaken as cohesive *Gardnerella*.

Occurrence and Distribution of Cohesive and Dispersed *Gardnerella* in 72 Pregnant Women and Their 72 Partners (table 1)

The occurrence rate of cohesive *Gardnerella* in unselected pregnant women was 17%. The Nugent score in 10 of the 12 pregnant women with cohesive *Gardnerella* was >6. Seven of the women reported complaints consistent with BV either presently or in the past. Five women were asymptomatic. The mean concentration of cohesive *Gardnerella* and the number of desquamated epithelial cells in pregnant women with accidentally detected cohesive *Gardnerella* were significantly lower than in patients with BV and comparable with those detected in unselected hospitalized patients positive for cohesive *Gardnerella*.

The occurrence of dispersed *Gardnerella* in pregnant women was 14%. All of these women had a Nugent score of ≤3. Two of the 53 women negative for *Gardnerella* had a Nugent score of >6, three had a Nugent score between 4 and 6, and all the others of ≤3.

The urine samples from partners of pregnant women positive for cohesive *Gardnerella* (n = 12) were either positive for cohesive *Gardnerella* (n = 8) or were nonanalyzable because of the low number of desquamated epithelial cells (n = 4). All male partners of pregnant women, who were negative for cohesive *Gardnerella* (n = 60), were also negative for cohesive *Gardnerella* (n = 60). There was no obvious congruence between findings of dispersed *Gardnerella* in partners of pregnant women.

Following the Chain of Infection

One of the BV patients participating in the longitudinal investigation of the urine samples had married for a second time 11 years ago. She reported that the BV complaints started with the second marriage. The husband from the first marriage was found negative for *Gardnerella*, while the second husband was positive for cohesive *Gardnerella*. The second husband was divorced from his first wife 15 years ago and has no children and has remained unmarried. The investigation of a urine sample from this woman proved positive for cohesive *Gardner-*

ella. The three children of the second marriage with both parents positive for cohesive *Gardnerella,* and living together in the same home, were all negative for *Gardnerella*.

The *G. vaginalis* strains were isolated from all three affected persons and sent for genotyping to Ghent, Belgium. The genotyping used arbitrarily primed PCR (RAPD) with fluorescent primer OPM1 followed by capillary electrophoresis. The strains of the husband, the current wife and the first wife divorced 15 years ago were identical.

Discussion

Our study demonstrates that adherent *Gardnerella* biofilm, which is characteristic for the condition presently known as bacterial vaginosis, can also involve a considerable proportion of the sexually active asymptomatic population, affect both females and males with the same frequency, and is sexually transmitted.

We investigated spontaneously voided urine for bacteria attached to desquamated epithelial cells in search of a reproducible and noninvasive test that would enable us to monitor adherent *Gardnerella* biofilms on vaginal epithelium and to replace vaginal biopsies in women with BV. We started by investigating BV patients for whom the presence of *Gardnerella* biofilm on the vaginal epithelium was established by vaginal biopsy. The results were encouraging. All urine samples from these BV patients contained large amounts of desquamated epithelial cells. The epithelial cells were covered with a thick layer of *Gardnerella* bacteria, which were tightly joined to structures resembling brickwork.

To check the relevance and the specificity of these findings, we investigated urine samples from a random population of hospitalized patients. In these patients, *Gardnerella* was found in two visually distinct forms: cohesive *Gardnerella* and dispersed *Gardnerella*. Dispersed *Gardnerella* was present in low concentrations in a subset of patients of both gender and age as isolated bacteria or as bacterial pairs without attachment to each other or to epithelial cells. Cohesive *Gardnerella* were tightly packed into groups of ≥100 bacteria and in a way more typical for bacterial vaginosis. Cohesive *Gardnerella* were detected in 13% of the unselected hospitalized women. Although the gynecologic complaints of these women were not specifically registered, the prevalence of cohesive *Gardnerella* observed in our study is in accordance with data on the epidemiology of BV reported previously [9]. To our surprise, 7% of unselected hospitalized male patients had also cohesive *Gardnerella* in their urine, which was visually in no way different to the findings observed in women with BV. This was in striking contradiction with the complete absence of cohesive *Gardnerella* in 50 hospitalized girls. The findings raised new questions: (1) Are cohesive and dispersed *Gardnerella* different entities or just due to a time-dependent shift in the concentration of the same bacterial population? (2) What is the habitat of *Gardnerella* in man? (3) Why can dispersed but not cohesive *Gardnerella* be found in children?

(1) To test whether cohesive and dispersed *Gardnerella* are possibly transitional states of the same condition, we investigated urine samples from two BV patients and from 20 healthy volunteers (male/female/child) daily over a period of 4 weeks. A shift from dispersed to cohesive *Gardnerella* and vice versa was not observed. All samples from patients with BV remained positive for cohesive *Gardnerella*. All samples from healthy controls remained negative for cohesive *Gardnerella*. Urine samples with dispersed *Gardnerella* from the same patient were not always positive for dispersed *Gardnerella*. Thus, cohesive *Gardnerella* proved to be stable over time, while the occurrence of dispersed *Gardnerella* was fluctuating.

(2) The concentrations of desquamated epithelial cells in the random hospital population were markedly lower in males than in females. While longitudinally investigating the urine samples from healthy male volunteers, we asked 3 of them to deliver two urine samples daily. One sample had to be obtained with the prepuce left over the glans penis, another with the prepuce pulled back. The number of desquamated epithelial cells in samples obtained with the prepuce covering the glans penis was comparable with those of the female population. The number of desquamated epithelial cells in urine samples from men with the prepuce pulled back was nearly zero and nonanalyzable for bacteria attached to the epithelial cells. Thus, the origin of desquamated epithelial cells in male urine samples is obviously the genital epithelium of the prepuce. From that point on we instructed the probands to leave the prepuce over the glans penis and to avoid cleaning the genitalia before voiding urine.

(3) The occurrence of cohesive *Gardnerella* in adult females and males but not in children could indicate sexual transmission of *Gardnerella* biofilms. Therefore, we investigated partners of the women with bacterial vaginosis and partners of random pregnant women who were routinely screened. All partners from women with BV were positive for cohesive *Gardnerella*. The occurrence of cohesive *Gardnerella* in random pairs awaiting childbirth

was 17% in females and 11% in males. Although the frequency of cohesive *Gardnerella* was higher in the female than in the male population, the sexual nature of transmission was apparent. The male partners of women, who were positive for cohesive *Gardnerella,* were either positive for cohesive *Gardnerella* or their urine samples were nonanalyzable because of the low number of epithelial cells. All female partners of men, who were positive for cohesive *Gardnerella,* were also positive for cohesive *Gardnerella*. All women negative for cohesive *Gardnerella* had partners negative for cohesive *Gardnerella*.

Starting our investigation we had no specific hypothesis to test, but were evaluating the distribution of the *Gardnerella* biofilm in different populations using a new developed method. During data collection, we were astonished over the clear but unexpected occurrence and transmission traits. We felt uneasy about the straightforward outcome of our own findings and therefore repeated single measurements on urine samples up to 5 times getting the same or even more distinct results each time. We were even able to follow-up the chain of infection in one case to a sexual contact that occurred 15 years previously. However, despite all progress, similar to Gardner [4], we are still not able to exactly define the nature of the pathogen. The bacterial vaginosis is not characterized by the presence of a definite microorganism, but is a result of bacterial biofilm formation on the epithelium of the vagina or prepuce. The main component of this biofilm is *G. vaginalis*. The detection of adherent *Gardnerella* conglomerates on the surface of vaginal biopsies or on the desquamated epithelial cells in urine samples is sufficient for diagnostic purposes. However, the in vivo detection of adherence is not sufficient to explain why *Gardnerella* builds biofilms in some patients and remains dispersed in others. The generally accepted explanation for lack of *Gardnerella* overgrowth in healthy subjects is bacterial antagonism of the 'normal' genital microbiota. We do not question the mechanisms of the bacterial overgrowth as those. However, we found no correlation between the presence of cohesive *Gardnerella* and the occurrence or concentration of *Lactobacillus* on desquamated epithelial cells of the urine samples (FISH data not shown). Additionally, the presence of cohesive *G. vaginalis* in both partners does not point to an important role for specific host immunity, which in this case has to be similar between both. We feel that the more probable explanation for biofilm formation is the specific property of some *Gardnerella* strains (plasmids, virulence factors, pathogenicity islands) or possible polymicrobial synergism, e.g. between *Gardnerella* and *Atopobium* species.

The presented FISH method analyzing urine sediment for *Gardnerella* biofilm is easy to perform, highly reliant and reproducible. Its expenses (as soon as a fluorescence microscope is purchased) do not differ from that of a Nugent score. Since the Carnoy-fixated samples can be stored practically indefinitely and sent by room temperature through the normal mail, the investigations can be performed in centralized laboratories making the costs for instrument, personnel and material even cheaper. Backed by present diagnostic possibilities, it should be a matter of time to isolate the strains responsible for biofilm formation and to investigate their properties. Whatever the outcome of these studies, it is now obvious that the presently used terminology, *G. vaginalis* and bacterial vaginosis, is insufficient. The correct name for *Gardnerella* biofilms should be gardnerellosis and a more appropriate name for the bacterial species might be *G. genitalis*.

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