FEMS IMMUNOLOGY & MEDICAL MICROBIOLOGY

RESEARCH ARTICLE

Desquamated epithelial cells covered with a polymicrobial biofilm typical for bacterial vaginosis are present in randomly selected cryopreserved donor semen

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Received 8 December 2009;accepted 9 April 2010.
Final version published online 21 May 2010.

DOI:10.1111/j.1574-695X.2010.00688.x

Editor: John Costerton

Keywords
polymicrobial biofilms; bacterial vaginosis; sexually transmittable disease; donor semen.

Abstract

We tested whether the bacterial biofilm typical for bacterial vaginosis (BV) can be found on desquamated epithelial cells in cryopreserved donor semen. Bacteria were detected with FISH. Bacterial biofilm, covering the epithelial layer in vaginal biopsies of 20 women with BV, was evaluated on desquamated epithelial cells found in the urine of these same women and their male partners ($N = 20$) and compared with the bacterial biofilm found on desquamated epithelial cells in randomly selected cryopreserved semen samples ($N = 20$). Urine from 20 healthy women of laboratory and clinic personnel and urine from their partners were used as controls. Desquamated epithelial cells covered with a polymicrobial *Gardnerella* biofilm were identified in urine samples from all women with BV and 13 of their male partners and in none of the female controls and their partners. *Gardnerella* biofilm, typical for BV, was found in the semen of three of the 20 donors. Donor semen might be a vector for BV.

Introduction

Bacterial vaginosis (BV) is often recalcitrant, leading to repeated visits of women to general and gynaecologic clinics. Antibiotic therapy brings relief, but the relapse rate within 6 weeks is high (Larsson & Forsum, 2005). Pathogenesis and mode and ways of propagation of the disease are not known, the data are contradictory and the scientific opinions are controversial (Sobel, 2000).

BV is not generally considered to be an infectious disease. However, of all the risk factors explored so far, the majority of epidemiologic studies have identified heterosexual, penetrative contact as the primary risk through a variety of outcome measures, most consistently lifetime number of sex partners, a recent history of multiple sex partners and a recent history of a new sex partner (Fethers *et al.*, 2008). Because BV epidemiology seems to mirror that of established sexually transmitted infections, it is tempting to suggest a sexual transmitted disease-like route of transmission.

Because a 46-year-old woman with recalcitrant BV reported that her vaginal complaints started after fertilization attempts through intrauterine insemination 4 years ago and continued since then, we explored whether intrauterine insemination could be a risk factor for disease. We wanted to know what exactly creates the risk of transmission.

We had previously investigated vaginal biopsies of women using FISH and shown that the vaginal epithelium in women with BV is covered with a polymicrobial biofilm (Swidsinski *et al.*, 2005). The main component of this biofilm was *Gardnerella vaginalis*. Multiple bacterial groups within the matrix of *G. vaginalis* could be found, of which *Atopobium vaginae* was the most frequent and numerous. Desquamated epithelial cells in women with BV were

covered *in situ* with an intact structured bacterial biofilm. In contrast, an adherent biofilm was absent in healthy women and the epithelial layer of the healthy vagina was practically free of bacteria (Swidsinski *et al.*, 2005). Bacteria attached to desquamated epithelial cells in healthy women and men were casually composed and probably represented saprophytic bacteria that degraded organic products of dead cells within vaginal secretions.

In this study, we assumed that the intact structure of a polymicrobial biofilm is necessary for transmission and we tested whether desquamated epithelial cells carrying an undamaged biofilm can be found in urine samples of women with BV and their partners, in healthy women and their partners and in random samples of cryopreserved donor semen.

Materials and methods

Subjects

(1) Urine from 20 women with biopsy confirmed *Gardnerella* biofilm and urine from their partners. All women had confirmed BV according to Amsel criteria and Nugent score and attended the Vivantes Clinics for Gynaecology as outpatients.
(2) Urine from 20 healthy women of laboratory clinic personnel and urine from their partners.
(3) Randomly selected cryopreserved semen samples from 20 men of infertile couples, which were collected, prepared and stored in the same way as sperm of sperm donors at the Kinderwunschzentrum in Dortmund, Germany.

Vaginal biopsies

Biopsies of about 3–5 mm diameter were taken from the middle side wall of the vagina with biopsy forceps (Aesculap, Tüttlingen, Germany), fixed in nonaqueous Carnoy solution (6/6/1 v ethanol/glacial acetic acid/chloroform) for 2 h, and then processed and embedded into paraffin blocks using standard techniques. Four micrometre sections were placed on SuperFrost Plus slides (R. Langenbrinck, Emmendingen, Germany) for FISH.

Urine

Fresh urine samples were fixated in Carnoy solution on the same day. An aliquot of 1.5 mL urine was centrifuged in a 1.5-mL Eppendorf tube for 6 min at 6000 ***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/6000 ***g***), decanted, 75 μL Carnoy solution was added and then the sample was stored at 4 °C.

For hybridizations, the 5 × 5 mm quadrant area of hybridization was marked with a PAP pen on a superfrost plus 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, dried for 30 min at 50 °C before the hybridization and then the sediments on the glass slides were incubated with 20 μL of 1% lysozyme for 15 min at 37 °C. The initial FISH analysis for this study was performed within 1 week after fixation of the urine sample. Some of the investigations were repeated up to 4 months later using Carnoy-fixated samples and yielded the same results.

The concentrations of epithelial cells in urine sediments were calculated per millilitre of 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 millilitre.

Sperm

Twenty microlitres of sperm were fixated in 200 μL of Carnoy solution. The hybridization of the Carnoy-fixated sperm sample was performed in the same manner as the hybridization of urine. The volume of aliquots taken for a single hybridization was usually 10–30 μL of the sperm–Carnoy mix. The hybridizations were performed until epithelial cells were detected or until the sperm sample was used up.

FISH

We used a Nikon e600 fluorescence microscope, Nikon DXM1200 camera and accompanying software (Nikon, Tokyo, Japan). The *Gardnerella* was assessed in a multicolour analysis using a mix of three probes: Bif164-Cy3/Eub338-FITC/GardV-Cy5 and DAPI counter stain. Other bacterial groups embedded within biofilm were assessed using group-specific probes as described (Swidsinski *et al.*, 2005).

Results

Vaginal biopsies

The 20 women with BV were selected because they had confirmed *Gardnerella* biofilm present in the vaginal biopsy (Fig. 1b). The biofilm was polymicrobial. The main components of the biofilm were represented by *Gardnerella*, followed by *Atopobium*, *Lactobacillus* ssp., *Bacteroides*, *Streptococcus*, *Corynebacteria* and *Enterobacteriaceae* (Swidsinski *et al.*, 2005). No biopsies were performed in healthy controls for ethical considerations, because biopsy results obtained

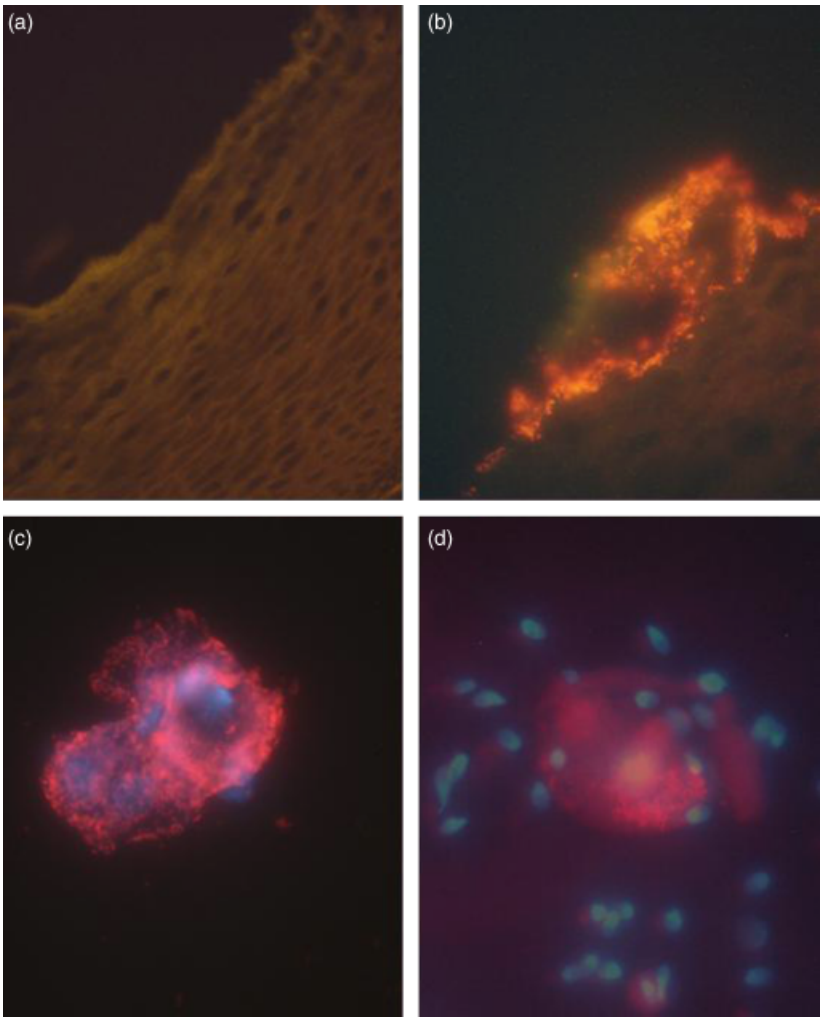

Fig. 1. (a) Vaginal epithelium from a healthy woman has no adherent bacteria when hybridized with the universal Eub338-Cy3 probe. No bacteria can be seen. The cell structures of vaginal epithelium can be clearly recognized due to the background fluorescence; this allows excellent orientation, magnification × 400. (b) Prolific polymicrobial biofilm covers the vaginal epithelium in a woman with bacterial vaginosis, seen in multicolour FISH using the *Gardnerella vaginalis* probe (GardV-Cy5 – red fluorescence) and *Lactobacillus* probe (Lab158-Cy3 – yellow fluorescence), magnification × 1000. (c) Biofilm composed mainly of *Gardnerella vaginalis* bacteria covers epithelial cells in the urine sediment of a woman with bacterial vaginosis, × 1000. GardV-Cy5 probe (red fluorescence) and DAPI stain (blue fluorescence) are overlaid. The large nuclei of epithelial cells can be seen clearly because DAPI has a high affinity to nucleic acid. Similar findings can be observed in the urine of male partners from women with bacterial vaginosis. (d) *Gardnerella* biofilm (GardV-Cy5 red fluorescence) is attached to an epithelial cell within a sperm sample from a potential donor, × 1000. The blue fluorescence of the epithelial cell nuclei and the nuclei of spermatozoa are seen clearly in the overlay with DAPI stain.

from healthy women were already evaluated and published previously (Swidsinski *et al.*, 2005).

Urine

Urine sediments of healthy women and women with BV contained significant amounts of epithelial cells with mean concentrations between 1.2 and $2.7 \times 10^4\,mL^{-1}$ (Table 1). The presence of epithelial cells in the urine sediment of male partners was significantly lower and depended on whether the foreskin was pulled over the glans penis during voiding. If the foreskin was pulled back for voiding or when men were circumcised, no epithelial cells could be found in the urine sediment. Therefore, we instructed all women included in this study how the urine samples in their male partner should be collected.

The desquamated epithelial cells in the urine sediments of all women with BV were covered with a bacterial biofilm similar to that observed in the vaginal biopsies of these women (Fig. 1b and c). Thirteen of 20 partners of women with BV had epithelial cells in the urine sediments, which were covered with *Gardnerella* biofilm, which was indistinguishable from the biofilm found in BV women. The other seven partners of women with BV had no epithelial cells in the urine sediments.

The desquamated epithelial cells in urine sediments of healthy women were either free of bacteria or were covered with unstructured loose accumulations of bacteria mainly composed of *Lactobacillus*, *Enterobacteriaceae* or *Enterococcus* groups. None had *Gardnerella* biofilm present. None of the male partners from healthy women had desquamated epithelial cells covered with *Gardnerella* biofilm in their urine samples. Desquamated epithelial cells in varying concentrations could be found in urine samples from all male partners of the healthy control women. This is different from the results in partners of women with BV, where no epithelial cells could be detected in the urine of seven partners. It may be that the compliance of the male partners

Table 1. Occurrence of epithelial cells with *Gardnerella* biofilm

	Material	Concentration of epithelial cells $mL^{-1} \times 10^4$ (mean ± SD)	No epithelial cells detectable	*Gardnerella* biofilm attached to epithelial cells
BV ($N = 20$)	Vaginal biopsies			20/20
	Epithelial cells in urine sediments	2.7 ± 2.9	0	20/20
	Epithelial cells in urine sediments of partner	0.6 ± 1.09	7/20	13/20
Healthy women ($N = 20$)	No vaginal biopsies performed			
	Epithelial cells in urine sediments	1.2 ± 1.8	0	0/20
	Epithelial cells in urine sediments of partner	0.2 ± 0.4	0	0/20
Randomly selected semen donor ($N = 20$)	Cryopreserved semen	Single cells	2/20	3/20

in the BV group, with regard to leaving the prepuce over the glans penis while voiding, was much lower than that of the controls, which where recruited from laboratory personnel and medical personnel of the hospital.

Sperm

Numerous spermatocytes were seen with DAPI stain, used to visualize all DNA structures, in all sperm samples. Despite repeated investigations and screening of large areas, loose bacteria lying between spermatocytes could not be identified in the hybridizations performed with the universal Eub338-Cy3 probes or any of the group-specific probes used. Obviously, the semen is bactericidal for bacteria.

The number of desquamated epithelial cells in the donor semen was anticipated to be low due to the swim-up technique, which is a standard procedure preceding cryopreservation aimed to remove unnecessary secretions, epithelial cells and leucocytes present in the native semen. After repeated investigations of aliquots of the semen samples, spread over large areas of the glass slide, intact epithelial cells could be found in 18 of the 20 samples. Single epithelial cells were seen between masses of spermatocytes in these 18 semen samples. The surface of epithelial cells in 14 of the 18 samples was free of bacteria. The epithelial cells were covered with a distinctive adherent and structured *Gardnerella* biofilm in three of the 18 donor sperm samples (Fig. 1d). In one sample, epithelial cells were covered with a bacterial biofilm that was clearly seen with DAPI stain, but the bacteria were not amenable to FISH evaluation with any of the tested bacterial probes probably due to suppressed metabolism and reduced number of ribosomes.

Discussion

We found *Gardnerella* in three of 20 randomly selected cryopreserved semen samples as a component of a complex polymicrobial biofilm attached to desquamated epithelial cells. These epithelial cells seem to be the true carrier of the *Gardnerella* biofilm.

A possible male involvement in the transition of *Gardnerella* as the putative causative agent in BV is backed by multiple data. Already in 1955, Gardner and Dukes isolated *G. vaginalis* from the urethra in 45 of 47 male partners of women with BV, and later Pheifer and colleagues detected *G. vaginalis* in 27 of 34 partners of BV patients (Gardner & Dukes, 1955; Pheifer *et al*., 1978). Although both studies did not include controls, the two largest cohort studies on male carriage of *G. vaginalis* conducted so far – both involving male attendees of a sexually transmitted disease clinic – documented male urethral carriage of *G. vaginalis* at rates of 11.4% (49/430) in the United Kingdom (Dawson *et al*., 1982) and 4.5% (10/309) in Sweden (Holst *et al*., 1984). The true male carriage of *G. vaginalis* may even be higher than estimated from the aforementioned studies, as urethral sampling is not optimal. Kinghorn *et al*. (1982) for instance found a significantly higher rate of *G. vaginalis* isolation from swabs taken from the prepuce than from urethral swabs. *Gardnerella* was also often isolated or detected through culture or PCR from semen samples (Chattopadhyay & Teli, 1984; Ison & Easmon, 1985; Elsner & Hartmann, 1987; Lam *et al*., 1988; Hillier *et al*., 1990; Kjaergaard *et al*., 1997).

Different from culture-backed studies, we found, using FISH, no relevant *Gardnerella* bacteria attached to spermatocytes or suspended in semen fluid as free-swimming bacteria or larger biofilm pieces. From the structural point of view, the semen itself seems to be an unlikely vector for the disease. Bacterial biofilm was found exclusively on desquamated epithelial cells, which were bymixed to the semen and obviously secondarily originating from the sloughing of the stratified squamous nonkeratinized epithelium, which is typical for vagina, foreskin, glanse penis and the distal urethra.

The second interesting finding of our study was the astonishingly clear relation of the *Gardnerella*-coated epithelial cells in the urine and BV. We detected *Gardnerella* biofilm-coated cells in all women with BV and 65% their male partners, but not in healthy female and their male

partners. Although seven of 20 male partners from women with BV had no epithelial cells in their urine samples, it does not automatically mean that they were biofilm free; they just did not shed sufficient number of epithelial cells in their urine that could be evaluated. Desquamated epithelial cells in vaginal secretions or urine originate mainly from the stratified squamous epithelium. When urine is voided with the foreskin pulled back from the glans penis, the surface of the squamous epithelium that is washed by the urine is very low and the number of desquamated epithelial cells in the urine declines below the level of detection. The probability of finding epithelial cells in donor semen is much higher than that in spontaneously voided urine. Masturbation precedes sperm donation. Usually, the man holds the penis (in erection) in his hand and moves the hand up and down. This leads to massive loosening of epithelial cells, which are then discharged with the sperm in large quantities. The swim-up technique, used for semen preparation and cryopreservation, separates spermatocytes from other components of the semen, reduces subsequently the number of admixed epithelial cells in semen, but does not eliminate them. Desquamated epithelial cells were still found in 18 of 20 investigated semen samples in our study. Circumcision reduces the rate of epithelial cells released at masturbation. However, circumcision is rarely performed in Germany and most European countries and cannot be taken as certain in the United States. In the United States, a national probability sample of adults in 1992, the National Health and Social Life Survey (Laumann *et al*., 1997) found that 77% of men reported being circumcised, including 81% of white men, 65% of black men and 54% of Hispanic men.

Finding of epithelial cells coated with a structured polymicrobial biofilm typical for BV in a subset of randomly chosen donor semen samples indicates that the *Gardnerella* biofilm could indeed be transferred to our 46-year-old woman with BV at the time of fertilization.

The possibility of BV being a sexually transmitted disease has a long history of arguments and counterarguments. Gardner & Dukes (1955) and later on Criswell *et al.* (1969) (were able to transmit BV using vaginal secretions of diseased women, but not when using a pure culture of *Gardnerella*. Criswell was able to induce BV through inoculation with overnight cultures of Gardnerella (Criswell *et al*., 1969), but these cultures could not be pure. The transmission of disease from men to women using *Gardnerella* culture was never shown. Koch's postulates, requiring transfection of isolated and a clearly characterized pathogen, were not fulfilled. Polymicrobial communities, however, cannot be transferred via single strains, but have to be transferred as a whole. In them, the different bacterial strains are not just accidentally associated, but assembled to a functional and structural community. The properties of single strains do not explain the properties of the biofilm.

One of the strongest objections against the sexually transmittable nature of the disease is an absence of an effect of antibiotic treatment of the male partner on the recurrence rate of BV in women (Moi *et al*., 1989; Potter, 1999). However, the same argument can be interpreted in terms of the overall low efficiency of the current antibiotic therapy. We have previously published a study on metronidazole therapy of BV based on FISH (Swidsinski *et al*., 2008). The study showed that the antibiotic treatment did not eradicate the bacterial biofilm, but made it metabolically silent. After withdrawal of the antibiotic, the biofilm recovered within weeks. As long as the 'eradicating' antibiotic therapy regimen is unknown, the failure of antibiotics to change the course of the disease does not really imply a lack of transmission, but rather a lack of therapy effectiveness. Another argument, challenging the infectious nature of BV, is a lack of an obvious pathogen, because *Gardnerella*, which is the main player in BV, could also be isolated in low numbers from a considerable proportion of healthy women and men. Thus, the presence of the *Gardnerella* in BV could just be a coincidence. The argument of lacking the specificity of *Gardnerella* isolation is correct in case of monoinfections, but not true with regard to polymicrobial biofilms. Clue cells, which are insensitive precursors of biofilm detection by non-FISH methods, are specific for BV and absent in healthy women.

Besides all the controversies regarding the nature of BV, the presence of epithelial cells covered with intact polymicrobial *Gardnerella* biofilm in a considerable portion of random donor semen is alarming. Mounting data indicate that BV is associated with an increased risk of sexually transmitted infections, including HIV, trichomoniasis, gonorrhoea, herpes simplex virus type 2 (HSV-2) and a vast disease burden of adverse obstetric outcome, including late foetal loss, preterm labour, spontaneous preterm birth and chronic pelvic inflammation (Jeff *et al*., 2009).

'Even though the presence of infectious organisms in cryopreserved semen does not alone assign an effective danger and many other interrelated factors need to exist for factual transmission (Mazzilli *et al*., 2006), it would be wrong to be complacent. This is not only necessary for fear of litigation, but it is an ethical issue.' It is our moral commitment to ensure that donated semen samples are free from potential health risks. It is within the range of investigative possibilities to discover *Gardnerella* biofilm attached to epithelial cells using the applied FISH technique. This method is reliable, easy to perform and cheap. Therefore, we recommend to screen urine sediment and semen from potential donors for *Gardnerella* biofilm and to discard such samples or at least to follow up the recipient. Women who were already inseminated with sperm containing epithelial cells covered with *Gardnerella* biofilm should be tested for *Gardnerella* biofilms. The *Gardnerella* strains from sperm and vagina can be compared with molecular-genetic

methods in order to prove that the infection in the woman came from the donor sperm.

Acknowledgements

This study was funded by University Funding (Charite Hospital, Berlin University), Bundesministerium für Bildung und Forschung (BMBF) network 'Resistance and Susceptibility to Intestinal Infections – an Integrated Network to Study the Interaction between Microbial and Host Factors'.

Authors' contribution

A.S., designed and performed the study, drafted the article, and approved the final version; Y.D., analysed and interpreted the FISH data, revised the manuscript and approved the final version; V.L.-B., substantial contribution to the design, drafting the article and revised it critically, approved the final version; W.M., provided the vaginal biopsies and urine samples from women with BV, helped draft the manuscript and approved the final manuscript; H.V., analysis and interpretation of data, revised it critically, and approved the final version; S.D., provided the semen samples, helped draft the manuscript and approved the final manuscript; J.S., organized and supervised collection and analysis of urine samples from healthy controls, analysed urine and semen samples, drafted the article and revised it critically, approved the final version.

Statement

The study was performed according to the ethical rules included in the Declaration of Helsinki. The investigations were approved by the Institutional Review Board of the Charité Universitätsmedizin Berlin and the patients gave informed consent.

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