

Response of *Gardnerella vaginalis* biofilm to 5 days of moxifloxacin treatment

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Introduction

Bacterial vaginosis (BV) is a common infection of the reproductive tract among women of childbearing age (Sobel, 2000). A marked increase in the risk of the acquisition of sexually transmitted diseases, the high frequency of preterm birth and other complications that are associated with BV have made the treatment of BV a global issue (Schwebke, 2003). Every gynecologist knows, however, how frustrating the treatment of BV is because of the low efficiency of the presently available therapies. Although the primary cure rates reported in the literature, based on the Nugent score (Nugent *et al.*, 1991) and Amsel criteria (Amsel *et al.*, 1983), were reported to be 60–70%, 20–30% of women with an initial response relapse within 3 months (Larsson & Forsum, 2005).

We could previously demonstrate a prolific bacterial biofilm adherent to the vaginal epithelium in women with

Abstract

Polymicrobial communities are often recalcitrant to antibiotics. We tested whether the polymicrobial *Gardnerella vaginalis* biofilm can be eradicated with moxifloxacin. Twenty women with bacterial vaginosis were treated with 400 mg moxifloxacin for 5 days. The changes in the occurrence and proportions of *Gardnerella*, *Atopobium* and *Lactobacillus* spp. were assessed using FISH. The bacterial biofilm was investigated using desquamated epithelial cells of spontaneously voided urine and sections of vaginal biopsies. Fifteen of 20 women showed a significant and sustained clinical response to moxifloxacin according to Amsel and Nugent criteria. The concentrations of adherent bacteria decreased significantly. The incidence and proportion of *Atopobium* declined sustainably. The proportions of *Lactobacillus* in the biofilm mass increased following therapy. Initially, *Gardnerella* was the main component of the polymicrobial biofilm. Following treatment, *Gardnerella* was not accessible to FISH in the urine and vaginal samples of 75% of all women. Ten to 12 weeks after the end of therapy, *Gardnerella* biofilm was cumulatively present in 40%. This was not due to newly acquired disease, but due to reactivation of the persisting, but biochemically inactive biofilm. Despite clear clinical efficacy, and initially definite suppression of the biofilm, moxifloxacin was, similar to metronidazole, not able to eradicate the *Gardnerella vaginalis* biofilm in all patients.

BV (Swidsinski *et al.*, 2005). The main component of the polymicrobial biofilm was *Gardnerella vaginalis*. Besides *Gardnerella*, *Atopobium vaginae* and *Lactobacillus* ssp. were most common and highly concentrated.

It is interesting to note that both main components of the biofilm, which are thought to be responsible for BV, *G. vaginalis* and *A. vaginae*, are theoretically not sensitive to metronidazole. Metronidazole is most effective in the case of Gram-negative anaerobes. *Gardnerella vaginalis*, however, is a Gram-positive bacterium of the *Bifidobacteriaceae* family. Although culture investigations of antibiotic resistance demonstrated the sensitivity of *G. vaginalis* to metronidazole, the studies performed on *Gardnerella*, growing as a biofilm, showed its resistance (Muli & Struthers, 1998).

We investigated the influence of metronidazole on the vaginal *Gardnerella* biofilm, and documented that in the short term, all women apparently converted successfully to

normal or intermediate vaginal microbial communities when using the Nugent score. The *Gardnerella* biofilm was only temporarily suppressed with metronidazole, and in most cases, recovered rapidly following treatment cessation (Swidsinski *et al.*, 2008).

Confronted with a low efficiency of metronidazole in eradicating the *Gardnerella* biofilm *in vivo*, we sought an antibiotic that could potentially be more successful. Of the newer oral antibiotics introduced, quinolones are distinguished by their enhanced spectrum of antimicrobial activity specifically against clinically important Gram-positive organisms and multidrug-resistant isolates as well as against anaerobes and atypical pathogens (Bolon, 2009). We chose moxifloxacin.

The adherent biofilm can be monitored longitudinally at the species level since the introduction of FISH. A typical representative of the vaginal biofilm is a bacterial coat that covers the clue cells and is seen in smears from women with BV. This bacterial coat of the clue cells can be analyzed directly using FISH. However, the vaginal smears are difficult to standardize for hybridizations with multiple FISH probes while vaginal biopsies cannot be taken repeatedly to monitor the effects of therapy. We therefore searched for a noninvasive method that would allow us to conduct longitudinal investigations and, for this purpose, we chose sediments of spontaneously voided urine containing large amounts of desquamated epithelial cells. 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. Carnoy-fixed urine sediment 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 longitudinal monitoring of the findings.

Materials and methods

Patients

Twenty women, 20–47 years old (mean age 32.7 years, SD 7.6 years), were enrolled. Women were included if they were diagnosed with BV as assessed by a Nugent score of at least 7 on Gram stain (Nugent *et al.*, 1991), were positive for four Amsel criteria (Amsel *et al.*, 1983) and if they had an adherent *Gardnerella* biofilm in the vaginal biopsy sections or on vaginal epithelial cells of urine sediment when investigated using FISH.

Treatment

All women were treated orally with moxifloxacin 400 mg day⁻¹ for 5 days.

Follow-up

Following treatment, the Amsel criteria and Nugent score of a Gram-stained vaginal smear and FISH of the vaginal biopsy were repeated at least once in all women during the 12 weeks of the study. To reduce the number of invasive investigations, each woman was investigated only twice with a vaginal biopsy. Women who could visit the practice provided one to three urine samples additionally for reassessment during the follow-up period. Controls were performed 5–7 days (week 1), 12–16 days (week 2), 21–28 days (week 3–4) and 10–12 weeks after the start of therapy.

All investigations were performed based on clinical reasoning and the medically indicated treatment of women. Because the women affected are mainly young and working, many of them could not visit the medical practice or even provide urine samples at will. Therefore, the attendance followed the usual rules of follow-up instead of defined controls points, leading to different follow-up times for each woman.

Biopsies

Biopsies of about 3–5 mm diameter were taken from the middle sidewall of the vagina using biopsy forceps (no. ER 058 RS; chubert, Aesculap, Tuttlingen, Germany) and were analyzed using FISH. They were fixated in non-aqueous Carnoy solution for 2 h, and then processed and embedded into paraffin blocks using standard techniques. Four μm sections were placed on SuperFrost slides (R. Langenbrinck, Emmendingen, Germany) (Swidsinski *et al.*, 2005).

Urine samples

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 at 6000 g), decanted, 75 μL Carnoy solution was added and then the sample was stored at 4 °C.

FISH analysis of urine sediments

A 5 × 5 mm quadrant area of hybridization was marked with a PAP pen on a superfrost glass slide. The Carnoy-fixed urine sediment was vortexed, and 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 before the hybridization. The sediments on the glass slides were incubated with 20 μL of 1% lysozyme for 15 min at 37 °C before hybridization.

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

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 differently stained group-specific and universal bacterial probes. The Eub338 probe represented virtually all bacteria, Kingdom (*Eu*)*Bacteria* (Amann *et al.*, 1990); GardV probe (*Gardnerella vaginalis*) derived from Bif 662 with 0 mismatches to *G. vaginalis* visualized *Gardnerella* (Swidsinski *et al.*, 2005); Ato291 was used to visualize *Atopobium* (Harmsen *et al.*, 2000); and Lab158 for the *Lactobacillus* groups (Harmsen *et al.*, 1999). The washing temperature was 50 °C. No formamide was used. A 4',6-diamidino-2-phenylindole (DAPI) counter stain was performed for each hybridization. Oligonucleotide probes were synthesized with a carbocyanine dye (Cy3), fluorescein isothiocyanate (FITC) or Cy5 fluorescent dye at the 5'-end.

Statistics

All statistical analyses were performed using the statistical software package SPSS v15.0 (Chicago, IL). The mean values and SDs were calculated from the bacterial counts. Using *t*-tests, a *P*-value of < 0.05 was considered significant.

Results

All 20 patients were positive for the four Amsel criteria and presented with a median Nugent score of 7.9 (interquartile range 7–9) at the initial assessment. The initial study of the vaginal biofilm characteristics at baseline, before treatment was initiated, was performed on a vaginal biopsy in all patients. In addition, vaginal epithelial cells isolated from a urine sample were studied in 13 women. All the results are given in Table 1.

No side effects of the therapy were observed during and after therapy.

After completion of the 5-day treatment course, 15 patients remained free of vaginal discharge, malodor and clue cells throughout the study period. In two women, the symptoms were not influenced by the therapy at any time. The symptoms recurred within 3 weeks in two women and within 12 weeks in one woman. In parallel to the clinical improvement, the vaginal pH, Amsel and Nugent scores also declined and remained significantly lower than the pretreatment values (Table 1).

The concentrations of adherent bacteria in the biopsy decreased significantly and remained low. The number of

bacteria adherent to desquamated vaginal epithelial cells in the urine was significantly decreased 2 weeks after therapy and increased slightly 10–12 weeks after the treatment. The amenability (accessibility) of the bacteria to the FISH probes declined drastically with the reduction of the number of adherent bacteria. During treatment, only $24 \pm 22\%$ of bacteria, which could be distinguished clearly in the DAPI stain, were amenable to, universal for all bacteria, the Eub338 probe. The amenability of bacteria to FISH probes increased after therapy, reaching values typical for the time before treatment, 10 weeks after the end of the treatment.

The percentage of women with *Atopobium* decreased from 70% before therapy to 8% 2 weeks after the start of treatment and remained low compared with the initial values (9–17%). Moxifloxacin caused little suppression of *Lactobacillus* occurrence. At the same time, the percentage of *Lactobacillus* of all detectable microbiota increased significantly immediately after moxifloxacin therapy and remained high thereafter.

Despite the clinical and bacteriological response, the elimination of adhesive *Gardnerella* was less obvious. The percent of women with adhesive *Gardnerella* declined to 25% 2 weeks after the start of moxifloxacin therapy; however, the percentage increased over time, reaching 40% after 10–12 weeks. The increase in the occurrence of *Gardnerella* was accompanied by a mean increase in the number of bacteria adherent to epithelial cells and an increase in the amenability of bacteria starting in the first week and completely recovered 10–12 weeks after therapy (Table 1).

When comparing samples from women who had a relapse of cohesive *Gardnerella* biofilm with successfully treated women, it could be clearly seen that the *Gardnerella* biofilm was not really eradicated, but persisted on the epithelial cells as a DAPI-positive, FISH-negative layer. In these women, the proportion of *Gardnerella* increased gradually after the end of the antibiotic treatment following the increase in the amenability of the biofilm (Fig. 1a–d).

Discussion

Our data demonstrate the clinical and microbiologic efficacy of moxifloxacin in the treatment of BV. A marked reduction of bacteria adherent to epithelial cells, a significant increase in the occurrence and proportions of *Lactobacilli* and a sustained reduction of *Atopobium* were achieved. Although the role played by single components in the pathogenesis of BV is unclear, it is generally accepted that *Lactobacillus* is positively while *Atopobium* is negatively correlated with BV (Sobel, 2000; De Backer *et al.*, 2007). From this point of view, the improvement of clinical symptoms demonstrated an effect of moxifloxacin on the microbiota adherent to vaginal epithelium.

Table 1. Clinical and bacteriological effects of moxifloxacin treatment

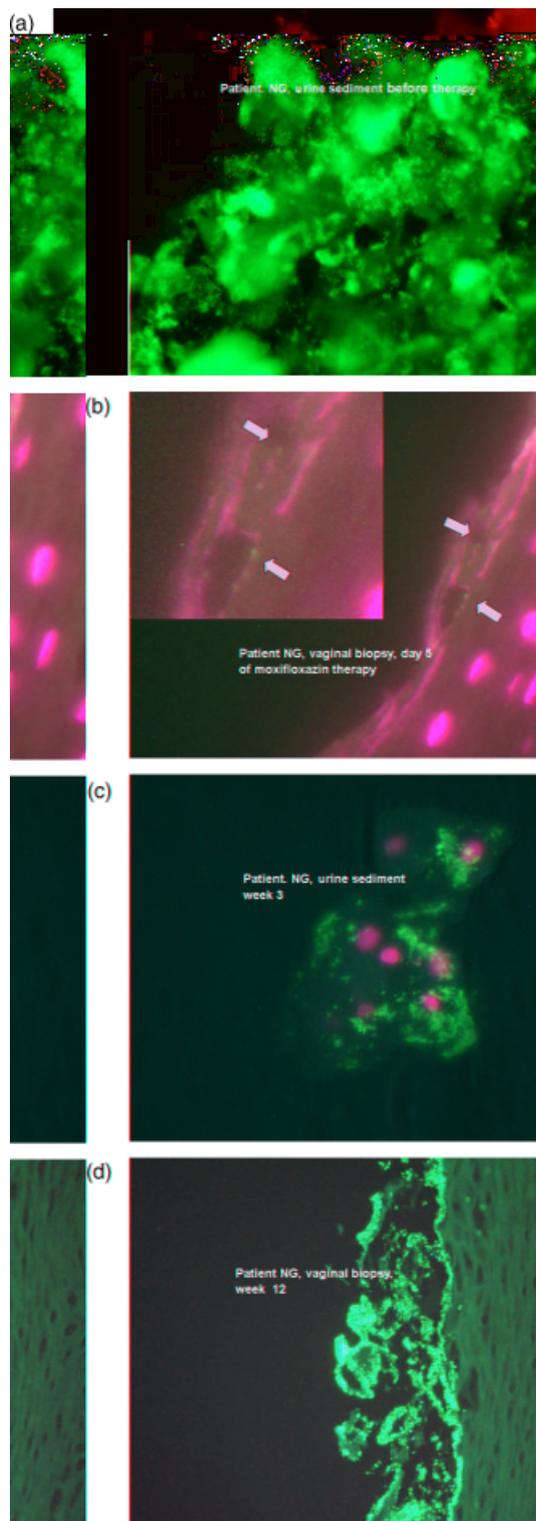
	Pretherapy	Therapy	Posttherapy			P
	A (n = 20)	B Week 1 (n = 17)	C Week 2 (n = 12)	D Week 3–4 (n = 11)	E Week 10–12 (n = 6)	
Vaginal pH (mean ± SD)	5.54 ± 0.25	4.56 ± 0.4	4.18 ± 0.19	4.51 ± 0.61	4.55 ± 0.44	A/B–E < 0.001
Percent of women with pH > 5	100 (20/20)	13 (2/15)	0 (0/9)	29 (2/7)	33 (2/6)	
Amsel criteria (mean ± SD)	4 ± 0	1.5 ± 1.4	0.5 ± 0.9	1.4 ± 1.9	1.3 ± 1.5	A/B–E < 0.001
Percent of women with Amsel ≥ 4	100 (20/20)	13 (2/15)	0 (0/9)	29 (2/7)	33 (2/6)	
Nugent score (mean ± SD)	7.92 ± 0.75	4.3 ± 2.1	1.9 ± 1.7	4.5 ± 3.2	3.5 ± 2.1	A/B–E < 0.001
Percent of women with Nugent score 0–3	0	33	75	43	66	
Percent of women with Nugent score 7	100 (20/20)	13 (2/15)	0 (0/9)	14 (1/7)	17 (1/6)	
Bacterial amenability (%) (mean ± SD)	77 ± 15	24 ± 22	51 ± 38	48 ± 33	72 ± 15	A/B–D 0.01–0.001 A/E NS
Bacterial concentrations within a vaginal biofilm (biopsy), (mean ± SD) × 10 ¹⁰ /mL vaginal contents according to DAPI stain	7.8 ± 2.8	0.25 ± 0.3	0.3 ± 0.8	0.3 ± 0.6	Not available	A/B–E < 0.001
Bacterial concentrations on vaginal epithelial cells in urine (mean ± SD) × 10 ⁴ /mL urine according to DAPI stain	7.8 ± 2.9	4.0 ± 7	0.04 ± 0.08	0.23 ± 0.39	1.5 ± 2.6	A/C–E < 0.001
Adherent <i>Gardnerella</i> , % of microbiota (mean ± SD)	71 ± 12	50 ± 38	15 ± 33	18 ± 33	28 ± 33	
Percent of women with adherent <i>Gardnerella</i>	100 (20/20)	74 (12/17)	25 (3/12)	28 (3/11)	40 (2/6)	A/B–E < 0.05–0.001
<i>Atopobium</i> , % of microbiota	13 ± 11	2 ± 5	< 0.1	< 1	4	A/B–D 0.01–0.001
Percent of women with detectable <i>Atopobium</i>	70	18 (3/17)	8 (1/12)	9 (1/11)	17 (1/6)	
<i>Lactobacillus</i> , % of microbiota (mean ± SD)	8.8 ± 8.5	8.9 ± 8.4	37 ± 38	22 ± 27	32 ± 33	A/C–E < 0.001
Percent of women with detectable <i>Lactobacillus</i>	90 (18/20)	76 (13/17)	75 (9/12)	73 (8/11)	100 (6/6)	
					100 (20/20)	

NS, not significant.

We must, however, state that despite these overall positive changes observed under moxifloxacin therapy, our specific goal to find a cure for adherent *Gardnerella* biofilm was not achieved in 40% of the patients and the relapse rate may have been higher if the women had been followed up for longer periods of time. Similar to metronidazole (Swidsinski et al., 2008), moxifloxacin suppressed adherent bacteria without eliminating them, leading to a 40% recurrence rate of the *Gardnerella* biofilm within 10–12 weeks after the end of therapy. We are not discouraged by the outcome of our study. Nearly half a century elapsed between the first description of monoinfections by Koch and Pasteur and efficient therapy. *Gardnerella* biofilms in bacterial vaginosis are polymicrobial. We are just starting to recognize the role played by polymicrobial infections in the pathogenesis of different diseases and describe new entities. Polymicrobials react differently to antibiotics than each of their compo-

nents (Keller & Costerton, 2009). Different lines of evidence indicate that organisms within polymicrobial communities are in symbiotic relationships for various metabolic requirements, which allows them to persist under conditions that would be deadly for each single species, and are resistant to antibiotic treatment. Our data confirm this. In contrast to the improvement of clinical symptoms, the eradication of adhesive polymicrobial *Gardnerella* biofilm proved to be difficult. Even antibiotic monotherapy with such potent drugs as metronidazole and moxifloxacin did not eliminate the biofilm in all cases. It is too early to predict when we will be able to control polymicrobial infections. Presently, we cannot even propagate a polymicrobial culture *in vitro*, which considerably hampers our possibilities of finding optimal therapy regimes and makes clinical studies based on calculated therapy unavoidable. Presently, there is no *in vitro* test that is predictive of the virtual effectiveness of

antibiotics on polymicrobial communities. We therefore refrained from *in vitro* antibiotic resistance testing of *Gardnerella* isolates.



Using FISH and other molecular genetic methods, we can now follow up the changes within polymicrobial communities continuously, thus improving and optimizing our initial therapy regimens. The following aspects should be considered when planning future trials:

- (1) Because of the recalcitrance of BV biofilms, a combination therapy rather than monotherapy should be applied and tested over different time periods.
- (2) The effect of therapy cannot and should not be evaluated exclusively clinically.
- (3) Because FISH does not allow to comparatively analyze the individual strains composing the biofilm, *Gardnerella* and *Gardnerella*-accompanying bacteria such as *Atopobium*/*Lactobacillus* should be isolated before and after treatment and compared genetically.
- (4) The overall low sensitivity of FISH to detect bacteria in low concentrations could be backed by microarray or real-time PCR technique, and the follow-up investigations should be performed for longer than 3 months.
- (5) We did not treat partners of women and it is probably too early to demand such a treatment. It is, however, necessary to monitor all partners for *Gardnerella* biofilms that are adherent to epithelial cells of the male prepuce and urethra during antibiotic therapy and thereafter. The sediments of spontaneously voided urine are perfect for this purpose.

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Fig. 1. Vaginal biopsies ($\times 400$) and urine samples ($\times 1000$) from the same patient with bacterial vaginosis. Hybridizations are performed using the GardV Cy5 probe (*Gardnerella*, red fluorescence). (a) Desquamated vaginal epithelial cells in urine sediment before treatment: showing vaginal epithelial cells covered with a prolific biofilm, containing mostly *Gardnerella vaginalis*. (b) Vaginal biopsies from the same women on the fifth day of moxifloxacin therapy. The *Gardnerella* hybridization signals are overlaid with DAPI stain, making the DNA structures visible. The adherent bacterial biofilm is still present and can be clearly seen in DAPI. However, the *Gardnerella*-specific probe hybridizes only with a small portion of the DAPI-positive cells (arrows). Most bacteria within the biofilm are not accessible to the FISH probes. (c) Desquamated vaginal epithelial cells in urine sediment three weeks after therapy. The *Gardnerella* hybridization signals are overlaid with DAPI stain, making the DNA structures visible and showing that the bacteria have recovered and hybridize well with the GardV probe. (d) Vaginal biopsies 12 weeks after therapy demonstrate that the biofilm has recovered completely.

Leverkusen, Germany, which funded the FISH investigations of the preexisting biopsies.

Conflict of interest: The FISH investigations of the preexisting biopsies were supported by a grant from Bayer Vital GmbH, Leverkusen, Germany.

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 provided informed consent.

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