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Dissimilarity in the occurrence of *Bifidobacteriaceae* in vaginal and perianal microbiota in women with bacterial vaginosis

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ABSTRACT

Recent data point at the similarity between the perianal and vaginal microflora in terms of *Lactobacillus* species involved. Bacterial vaginosis, the most common perturbation of the vaginal microflora involving primarily overgrowth of *Gardnerella vaginalis*, has also been suggested to involve a recto-vaginal pathway. We addressed this issue with regard to bacteria of the *Bifidobacteriaceae* family. In particular, we investigated the putative concordance of the presence of *G. vaginalis* and a series of *Bifidobacteria* between the perianal and vaginal microflora in 10 patients with bacterial vaginosis through multicolor fluorescence in situ hybridization analysis of desquamated epithelial cells.

G. vaginalis was found in a biofilm mode of growth at the perianal and vaginal sites. In most women at least one of the following species was detected perianally: *Bifidobacterium adolescentis, Bifidobacterium longum, Bifidobacterium breves, Bifidobacterium bifidum* and *Bifidobacterium catenulatum*. At the vaginal site, none of these *Bifidobacteria* was found.

We conclude that bacterial vaginosis does not occur as a result of simple growth per continuum of perianal bacteria. Only some species originating from the intestinal tract do display pronounced vaginotropism, like *G. vaginalis*, whereas many other species do not.

1. Introduction

Bacterial vaginosis (BV) is a common condition characterized by massive overgrowth of the vaginal epithelium with a wide variety of anaerobes [1,2]. This anaerobic overgrowth primarily involves proliferation of *Gardnerella vaginalis* in a biofilm mode of growth forming dense layers of stacked bacteria adherent to the vaginal epithelium [3]. The epidemiology of *G. vaginalis* remains elusive. *G. vaginalis* is commonly found in relatively low concentrations with normal lactobacilli-dominated microflora in the vagina, possibly originating from an intestinal reservoir. In a large cohort study, Holst isolated *G. vaginalis* from the rectum in 45% of 148 women with BV, while *G. vaginalis* occurred at a significantly lower 10% rate in the rectum of 69 women without BV [4]. A recent PCR-based study also pointed at a marked similarity between rectal and vaginal microflora in with regard to *Lactobacillus* species [5].

This relationship has not been studied with regard to *Bifidobacteriaceae* other than *G. vaginalis*, a large family of Gram-positive bacteria including the *Bifidobacteria*, which is one of major genera constituting the intestinal microflora. Rosenstein et al. however found that *Bifidobacterium* spp. appeared early with normal (grade I) vaginal microflora converting to abnormal microflora (grade I revertants), and continued to increase in grades II (intermediate microflora) and grade III (bacterial vaginosis), while other species, such as G. *vaginalis* and *Mycoplasma hominis*, were only found in large numbers the late stage of full-blown bacterial vaginosis [6]. In particular, in the later study, *Bifidobacterium* spp. were found in 12% of healthy controls, in 41% of grade I revertants, in 58% of those with intermediate microflora and in 94% of patients with bacterial vaginosis. Hence, from this study, it seems as if *Bifidobacterium* spp. are gradually involved in the conversion from normal to bacterial vaginosis microflora, which in turn, may implicate a rectal-vaginal pathway in the pathogenesis of bacterial vaginosis.

Abbreviations: FISH, fluorescence in situ hybridization; *Cy3, FITC, Cy5, DAPI*, Different fluorescent dyes corresponding to orange, green, dark red, and blue colors.

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***FISH probes abbreviations used in text in relation to bacteria targeted*:**

Bif164	*Bifidobacteriaceae, Bifidobacterium* spp.
Bif153	Genus *Bifidobacterium*
GardV	*Gardnerella* and *Bifidobacteria*
Bif1278	*Bifidobacterium* spp.
Bif662	*Bifidobacterium* spp.
Bden82	*B. dentium*
Bifado182	*B. adolescentis*
Bbif186	*B. bifidum*
Bcat187	*B. catenulatum group*
Bang198	*B. angulatum*
Bbrel198	*B. breve*
Bifado434	*B. adolescentis*
Blon1004	*B. longum*
Eub338	Eubacteria, virtually all bacteria

Conversely, it may be hypothesized that *Bifidobacterium* spp. present in the vagina along with the *G. vaginalis* biofilm could colonize simultaneously the perianal and anal epithelium. In particular, it has recently been suggested that a biofilm facilitates the transmission of pathogens by providing a stable protective environment and by acting as a nidus for the dissemination of microorganisms [7]. The vaginal and anal/perianal epithelia are not only anatomically adjacent but also histologically similar, suggesting that bacteria that are able to adhere to vaginal epithelium could also adhere to anal/perianal epithelium. We therefore sought to test whether the composition of *Bifidobacteria* in the vaginal and perianal biofilms are similar in women with BV through fluorescence in situ hybridization (FISH) with species-specific FISH probes for 11 different *Bifidobacteria* in 10 women diagnosed with BV.

2. Materials and methods

2.1. Subjects

Ten women from the Outpatient Clinic of the Charité Hospital, Berlin, Germany, with symptomatic BV, diagnosed according to the Amsel criteria [8]and Nugent score [9], were included. The study was approved by the local Institutional Review Board and all patients gave written and oral informed consent. Desquamated epithelial cells from the vagina and anus were investigated for the presence of adherent bacteria using FISH as previously described [3,10]. Voided urine was collected and vortexed to obtain desquamated vaginal epithelial cells, which are expelled from the vagina during urination. We have previously shown that voided urine is a non-invasive method for obtaining epithelial cells, bypassing the need for vaginal biopsies [16].

Previously vaginal epithelial cells were mainly investigated using smears of vaginal swabs. The FISH analysis of the bacterial community, however, requires multiple hybridizations, which should be carried out under exactly reproducible conditions. This 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 non-invasively, replacing the vaginal biopsy investigations. The numbers of epithelial cells from the urinary tracts are usually low, not covered by biofilms and therefore not interfering with the analysis. We chose urine samples for practical reasons after comparative pilot investigations have shown its practicability. Both sessile bacteria (attached to the epithelial surface) and bacteria suspended in urine can be investigated. Carnoy fixated urine sediments 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.

Desquamated epithelial cells from the perianal/anal region were obtained from the same patients using adhesive tape applied over the anus.

2.2. Preparation of desquamated vaginal epithelial cells

Fresh samples of spontaneously voided urine were fixated in Carnoy solution (ethanol/glacial acetic acid/chloroform in a 6/6/1 volume ratio). An aliquot of 1.5 ml urine was centrifuged in a 1.5 ml Eppendorf tube for 6 min at 6000 G. The supernatant was removed and the sediment resuspended with 1 ml of Carnoy solution and left at room temperature to incubate. After 60 min, the sediment was centrifuged once more (6 min at 6000 G), decanted, 75 μl of 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 of fixation of the urine sample.

For FISH analysis, a 5 × 5 mm quadrant area of hybridization was marked with a PAP pen on a superfrost glass slide. Prior to the hybridization, 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, and then the sediment on the glass slide was incubated with 20 μl of 1% lysozyme for 15 min at room temperature.

2.3. Preparation of desquamated epithelial cells from the perianal/anal region

The buttocks were spread apart and a 2 × 2 cm piece of adhesive tape was attached over the anus of the patient for approximately 10 s. The adhesive tape was then removed and placed in 1 ml of Carnoy solution. After 24 h of fixation, the tape was embedded in paraffin. The embedded tape was histologically processed into longitudinal strips. Epithelial cells were also found in the sediment of the Carnoy solution in which the adhesive tape had been fixated. The perianal/anal epithelial cells were collected, fixated, and hybridized, similar as previously described above for the urine sediment.

2.4. FISH analysis

We used a Nikon e600 fluorescence microscope, Nikon DXM1200 camera and accompanying software (Nikon, Tokyo, Japan). Altogether 11 species-specific Bifidobacteria probes were used, as well as one *Gardnerella*/Bifidobacteria probe (Table 1). As the *G. vaginalis* probe cross-hybridized with other Bifidobacteria species, positive hybridization with GardV was validated by identification of *G. vaginalis* both phenotypically as well as with sequencing of 16s RNA. Bifidobacteria species were assessed in a multicolor analysis using a mix of three probes: universal Eubacteria Eub338 probe marked with FITC (green fluorescence) to visualize all bacteria, Bifidobacteriaceae Bif164 probe marked with Cy5 (dark red fluorescence) to visualize bacteria of the Bifidobacteriaceae family, and one of the 11 group specific Bifidobacteria probes or Gardnerella/Bifidobacteria probe marked with Cy3 (orange fluorescence) to visualize particular Bifidobacteria species [12]. Nuclei of epithelial cells were stained with DAPI counter stain. The conversion of the numbers of bacteria within a microscopic field to concentration of bacteria per ml was based on the calculation that a 10-μl sample with a cell concentration of 10^7 cells per

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Table 1
FISH probes used in the study.

Probe.	Bacterial Group.
Bif164	Bifidobacteriaceae, *Bifidobacterium* spp
Bif153	Genus *Bifidobacterium*
GardV	*Gardnerella* and *Bifidobacteria*
Bif1278	*Bifidobacterium* spp
Bif662	*Bifidobacterium* spp
Bden82	*B. dentium*
Bifado182	*B. adolescentis*
Bbif186	*B. bifidum*
Bcat187	*B. catenulatum group*
Bang198	*B. angulatum*
Bbrel198	*B. breve*
Bifado434	*B. adolescentis*
Blon1004	*B. longum*
Eub338	Eubacteria (virtually all bacteria)

ml has 40 cells per average microscopic field at a magnification of 1000, the details of this conversion were previously described [11].

Concentrations of epithelial cells in urine sediments were calculated per mL of urine. The numbers of adherent bacteria were enumerated per epithelial cell (maximal and mean per sample). The concentrations of adherent bacteria in urine were obtained by multiplication of mean numbers per epithelial cell with concentrations of epithelial cells per mL.

3. Results

3.1. Vaginal epithelium

The urine samples of the women with bacterial vaginosis contained desquamated epithelial cells at concentrations ranging from 0.9 to 7.8 $\times$ 10^4 cells/mL. The cells were covered with a prolific *Gardnerella* biofilm (Fig. 1A). The local concentration of *Gardnerella* within this biofilm reached as high as 3 $\times$ 10^{11} cells/mL, with bacteria tightly packed in biofilm layers.

Although the biofilm contained many other different bacterial species that hybridized with the universal bacterial Eub338 probe in absence of hybridization with the GardV probe (not shown), none of the other *Bifidobacteria* species (underlined in Table 1) were detected within the biofilm in the patients with BV.

3.2. Anal/perianal epithelium

The strips of adhesive tapes from the perianal/anal region were covered with bacteria and single desquamated epithelial cells following the fade line of unspecific fluorescence left in place of the adhesive layer. Desquamated epithelial cells were also found in high numbers in the sediment of the Carnoy solution, in which the tape was fixed previous to being embedded in paraffin. Obviously, the Carnoy solution dissolved the adhesive layer of the tape and a significant number of epithelial cells were dispersed in the Carnoy solution. In 8 of 10 women evaluated, at least one of the *Bifidobacteria* species including *Bifidobacterium adolescentis*, *Bifidobacterium longum*, *Bifidobacterium breves*, *Bifidobacterium bifidum* and *Bifidobacterium catenulatum* could be detected both on the surface of the adhesive tape (Fig. 2A) as well as in the Carnoy solution (Fig. 2B,b) in noticeable quantities.

No positive hybridization signals were observed with the Bden82 (*Bifidobacterium dentium*), Bifado182 (*B. adolescentis*), Bang198 (*Bifidobacterium angulatum*), Bbrel198 (*Bifidobacterium breve*) probes either during the evaluation of the anal/perianal or vaginal epithelium. The hybridization signals generated by the

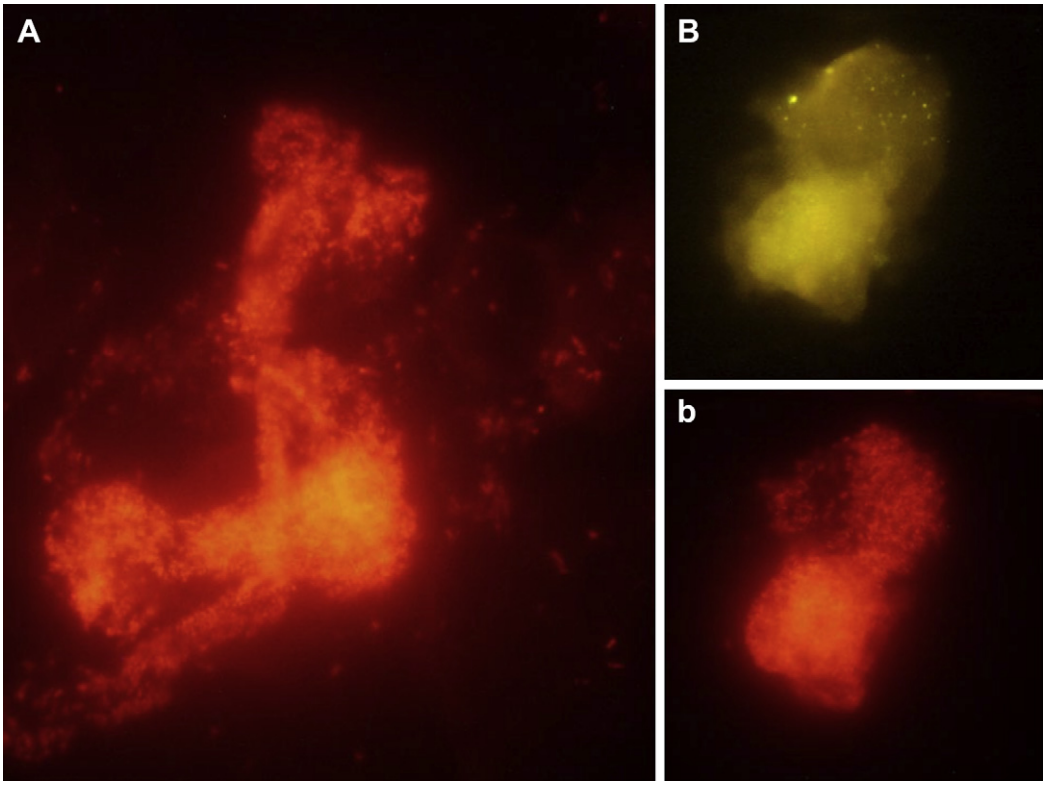

Fig. 1. A Conglomerate of desquamated epithelial cells covered with prolific *Gardnerella* biofilm in urine sediment from women withg BV (GardV-C5 probe, red fluorescence, x 1000). B(b) Perianal epithelial cell hybridized in multicolor FISH using the Blon1004-Cy3 probe (*B. longum*, orange fluorescence) and Bif164-Cy5 (*Bifidobacteriaceae*, red fluorescence). One can clearly see that *B. longum* composes only a small portion of the bacterial biofilm attached to the perianal epithelial cell. The matrix is composed primarily of *Gardnerella* bacteria, while *Bifidobacterium longum* is dispersed and produced no conglomerates of bacteria.

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GardV probe and the Bif164 and Bif662 probes universal for all *Bifidobacteriaceae*, were difficult to distinguish from each other, since the GardV probe cross-hybridized with other bacteria from the *Bifidobacteriaceae* group.

Bacteria positive with the GardV probe and attached to the perianal epithelial cells were building confluent bacterial biofilms with bacterial cells packed in a similar way as was observed in epithelial cells from urine sediments (Fig. 1B).

The occurrence and concentration of single *Bifidobacteria* species on the surface of the adhesive tape taken from the perianal region are represented in Table 2. The local concentrations of *B. adolescentis* and *B. longum* were the highest. Both species composed up to 30% of all *Bifidobacteria* species in some of the women.

However, none of these species produce confluent layers or conglomerates of bacteria either on the surface of the adhesive tape (Fig. 2A) or on desquamated perianal epithelial cells (Fig. 2B/b), but were either homogenous or locally focused by mixtures.

Table 2
Occurrence and local concentrations of single *Bifidobacteria* species on epithelial cells in anal/perianal biofilms $\times 10^9$ cfu/ml.

subject	GardV	Bif434	Blon1004	Bbif186	Bif1278	Bcat187
1	12	4	4		2	0,1
2	4	8			>0	
3	12	6	1		2	
4	6					
5	10	4	8		3	
6	8					
7	8	0,1		1		
8	5	2				
9	12	0,2		0,01		
10	2	2	2			

4. Discussion

Increasing evidence supports the similarity between the vaginal and rectal microflora. As *Bifidobacterium* spp have been found to be gradually involved in the conversion from normal vaginal microflora to bacterial vaginosis [6], possibly acting as initiating pathogens, and since many bifidobacteria are indigenous to the intestinal tract, we aimed to investigate whether such concordance could also be demonstrated for a series of species belonging to *Bifidobacteriaceae* in perianal and vaginal biofilms.

Our data demonstrated however a marked difference in occurrence and concentrations between the anal/perianal and vaginal *Bifidobacteriaceae* in women with bacterial vaginosis indicating an absence of a specific bacterial biofilm connecting these two anatomic regions. Although five *Bifidobacteria* species (*B. adolescentis, B. longum, B. breve, B. bifidum* and *B. catenulatum*) were found with specific probes perianally in concentrations comparable to those seen with the group specific (*G. vaginalis/Bifidobacteriaceae)* GardV probe, none of these species could be detected on desquamated vaginal cells of the same women at the resolution level achieved using FISH.

While *G. vaginalis*, formed cohesive, prolific biofilms on the surface of desquamated epithelial cells, other Bifidobacteria species found, were not adherent and were found dispersed and diffusely distributed between other bacteria within the perianal biofilm.

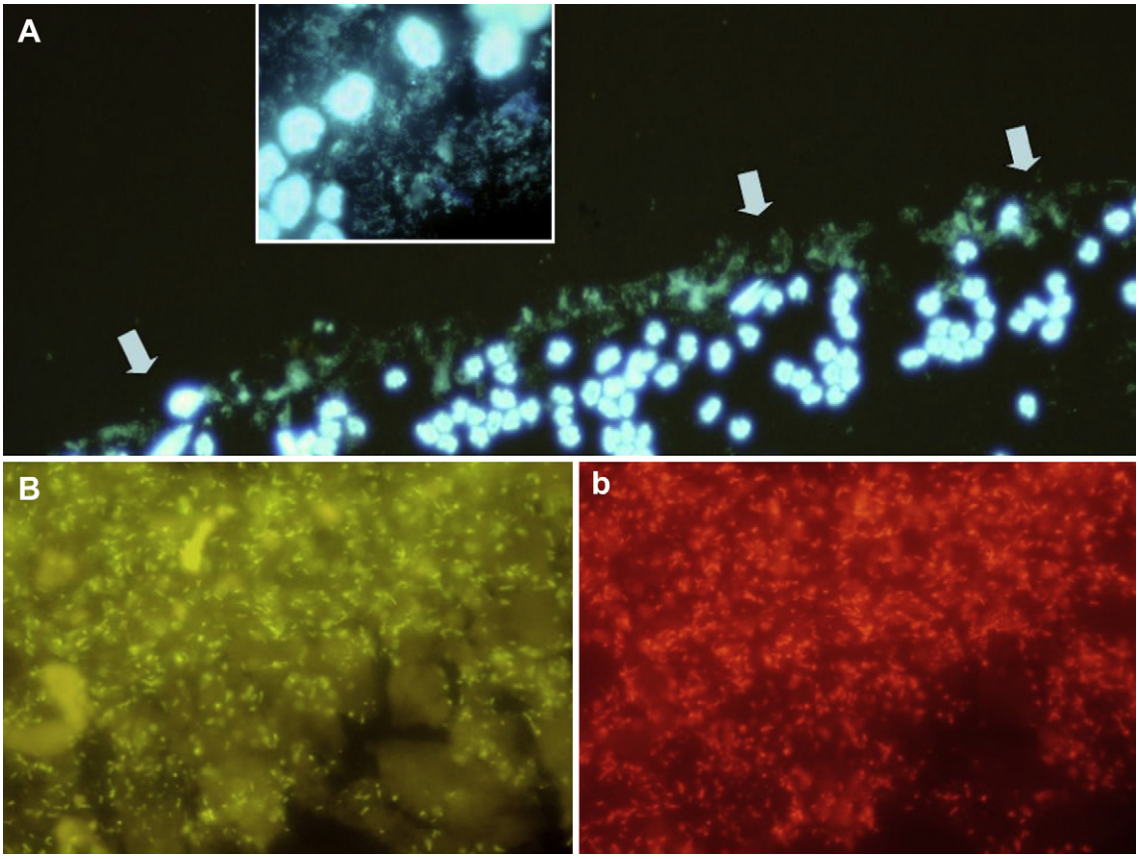

Fig. 2. A DAPI stain of a cross section of adhesive tape which was applied perianally at a low magnification of 100. Abundant bacteria can be seen attached to the surface of the tape (arrows), which can be better recognized at higher resolution of 400 (insert). B(b) Multicolor hybridization with (B) Bifido434-Cy3 [orange fluorescence, *B. adolescentis*] and (b) Bif164-Cy5 probe [*Bifidobacteriaceae,* red fluorescence] at magnification of 1000. Although *B. adolescentis* composes 30% of the *Bifidobacteriaceae* it is diffusely dispersed and does not co-aggregate.

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Burton et al. previously characterized the presence of vaginal bifidobacteria through denaturing gradient gel electrophoresis (DGGE) and thereby targeted 21 bifidobacterial strains belonging to 11 species with bifidobacteria-specific primers [13]. Based on their study, the authors concluded that bifidobacteria were infrequently detected indicating that they are not common components of the normal vaginal bacterial microbiota, and that these bifidobacteria may be fecal in origin, given the species detected [13]. Hyman et al. characterized the vaginal microbiota of 20 women – for which no data on vaginal microflora status were given – through cloning and sequencing of 16S rDNA gene, and found that one of the 20 woman had a vaginal microflora dominated by *Bifidobacterium* species, while another subject presented with *Bifidobacterium breve*, *Bifidobacterium* sp. oral strain H6-M4, and with *Bifidobacterium urinalis* alongside *L. gasseri* as the predominant species [14]. Verhelst et al. even described a specific vaginal microflora category termed 'grade I-like' characterized by the presence of diphtheroid bacilli cell types on Gram stain, and established that half of these samples contained Bifidobacterium spp, primarily *B. breve* [15]. Intermediate microflora in this study was to a limited extent represented by *B. breve*, whereas some bacterial vaginosis samples showed *B. bifidum*, *B. breve*, *B. dentium,* and/*or B. longum* [15].

It must be acknowledged that contrary to the above mentioned PCR-based detection methods, the sensitivity of FISH is lower. Spreading 10 μL of a 10^7 suspension of bacteria over a region of 1 cm leads to only 40 bacteria in a single microscopic field. When spreading a bacterial suspension with 10^3 bacteria per mL, it is necessary to evaluate 2.5 microscopic fields to find a single bacterial cell. FISH has however a unique advantage, since it allows to directly visualize bacteria in relation to each other and epithelial cells of the host. Any relevant accumulation of bacteria, which is needed for biofilm formation, can be assessed and confluent biofilm cannot be overlooked. Our data therefore clearly indicate, that only *G. vaginalis* and not Bifidobacteriaceae which are also present perianally in high concentrations contribute to the composition of the vaginal biofilm.

This observation is also evidence that bacterial vaginosis does not occur as a result of simple growth per continuum of perianal bacterial biofilm migrating to the vagina. Rather it seems as if some species originating from the intestinal tract do display pronounced vaginotropism, like *G. vaginalis*, whereas many other species do not.

Ethical approval

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

Authors' contributions

Alexander Swidsinski – designed and performed the study, drafted the article, and approved the final version.

Jennifer L. Patterson – analysed and interpreted the FISH data, revised the manuscript and approved the final version.

Vera Loening-Baucke – substantial contribution to the design, drafting the article and revised it critically, approved the final version.

Werner Mendling – provided the urine samples from women with BV, helped draft the manuscript and approved the final manuscript.

Hans Verstraelen – analysis and interpretation of data, drafting the article, revised it critically, and approved the final version.

Yvonne Dörffel – provided the urine samples from women with BV, helped draft the manuscript and approved the final manuscript.

Johannes Schilling – organized and supervised collection and analysis of urine samples and perianal samples, analysed FISH, drafted the article and revised it critically, approved the final version.

Conflict of interest

None.

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