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Increased *Gardnerella vaginalis* urogenital biofilm in inflammatory bowel disease

Johannes Schilling[a,b], **Vera Loening-Baucke**[a], **Yvonne Dörffel**[b,*]

[a] *Laboratory for Molecular Genetics, Polymicrobial Infections and Bacterial Biofilms, Charité Universitätsmedizin Berlin, Campus Mitte, 10117 Berlin, Germany*
[b] *Outpatient Clinic, Charité Universitätsmedizin Berlin, Campus Mitte, Luisenstr. 11-13, 10117 Berlin, Germany*

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Abstract

Introduction: Inflammatory bowel disease (IBD) is a systemic inflammatory condition that affects the entire organism, not only the bowel. An impaired interaction with microbiota has been shown to be important. We looked for bacterial factors, which may contribute to the well-known higher incidence of poor reproductive outcome in IBD.
Methods: Urine specimen of patients with Crohn's disease (N = 42), ulcerative colitis (N = 46), and randomly selected patients attending the General Internal Medicine Outpatient Clinic of the Charité for non-IBD related medical conditions (N = 49) was analyzed for bacteria adherent to desquamated epithelial cells and diffusely distributed bacteria in the urine using fluorescence in situ hybridization.
Results: The urine of IBD patients contained significantly more often *Gardnerella vaginalis* biofilms (CD 38%, UC 43%) than those of the control group (16%). There was no link between current disease activity, history of and present fistula and *G. vaginalis* biofilms, but the samples of patients with steroid refractory/dependent disease were significantly more often *G. vaginalis* biofilm positive. No significant differences in number of epithelial cells and leukocytes, and total bacterial counts were present.
Conclusions: There is a significant link between IBD and *G. vaginalis* biofilm. This observation suggests an epithelial barrier dysfunction of the genital tract. Since *G. vaginalis* is believed to be one of the reasons responsible for bacterial vaginosis, it may be an important factor in the well-known higher incidence of poor reproductive outcome in IBD. Excessive *G. vaginalis* biofilms in steroid refractory/dependent disease suggests a need to avoid long-term steroid therapy.

Abbreviations: A. vaginae, *Atopobium vaginae*; BV, bacterial vaginosis; CAI, colitis activity index; CD, Crohn's disease; CDAI, Crohn's disease activity index; Cy3, cyanine dye, yellow fluorescence; Cy5, cyanine dye, red fluorescence; DAPI, 4′,6-diamidino-2-phenylindole; FISH, fluorescence in situ hybridization; FITC, fluorescein isothiocyanate; *G. vaginalis*, *Gardnerella vaginalis*; IBD, inflammatory bowel diseases; UC, ulcerative colitis.

* Corresponding author at: Outpatient Clinic, Charité Universitätsmedizin Berlin, Campus Mitte, Luisenstrasse 11-13, 10098 Berlin, Germany. Tel.: +49 30450514018; fax: +49 30450514912.
E-mail address: yvonne.doerffel@charite.de (Y. Dörffel).

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1. Introduction

The structure of intestinal microbiota differs in patients with inflammatory bowel disease (IBD) and healthy and disease controls.[1] We and others have previously reported on changes of the intestinal microbiota in patients with IBD.[1–4] IBD affects the entire organism, not only the bowel. Till now, most studies on microbial structure in IBD patients were carried out on feces. Little is known about urogenital microbiota in patients with IBD.

Williams et al.[5] showed that specific urinary metabolites, such as hippurate and formate levels related to gut microbial metabolism, differ between patients with Crohn's disease (CD), patients with ulcerative colitis (UC), and controls. It is possible that there are specific changes in the composition of the urogenital microbiota in patients with IBD. Bacteria are commonly found in relatively low concentrations in the urine sediment. Many epithelial cells are found in spontaneously voided urine and bacteria adherent to them can be investigated noninvasively. We found that urine samples from women with bacterial vaginosis gave similar results as vaginal biopsies.[6] In addition, Dong et al.[7] described the reliability of the urine samples of the initial void in the analysis of the microbial communities in the distal male urethra. They showed that the microbiota in male's initial voided urine and the urethral swab specimens were nearly identical. When we characterized the spatial distribution of bacteria adherent to the urethral epithelium of the initial voided urine from adult men using fluorescence in situ hybridization (FISH) analysis, we found that *Gardnerella vaginalis* biofilms are present in 7% of the samples of unselected hospitalized male patients.[6]

G. vaginalis is a gram variable rod that is believed to be the most important pathogen in bacterial vaginosis. It exists in a dispersed and in a biofilm mode. The dispersed form is unspecific, while the biofilm mode is indicative for bacterial vaginosis (BV).[6,8]

A vast number of data published over the last 30 years demonstrate that BV is associated with high risk preterm birth. We had shown previously that *Gardnerella* dominated BV biofilms and can spread up to the uterine cavity and infect the fetus, possibly explaining this association.[9]

Since a high preterm birth rate had been also described in CD women, we hypothesized that the disturbance of the vaginal flora may be an explanation for the higher preterm birth rate in CD patients.

2. Materials and methods

2.1. Patients

Forty-two patients (30 females and 12 males) with histologically confirmed CD, 46 patients (31 females and 15 males) with histologically confirmed UC, and 49 patients (37 females and 12 males) (controls), who attended the General Internal Medicine Outpatient Clinic of the Charité for other reasons than IBD, were enrolled. Participants with current antibiotic treatment or current urinary tract infections (UTIs) were excluded.

The current disease activity in IBD patients was rated according to the Crohn's disease activity score (CDAI)[10] and the Colitis activity score (CAI).[11] A CDAI of $\geq$150 indicated active CD, while a CAI of $\geq$5 indicated active UC. IBD was counted as severe, when the CDAI score was >450 or the CAI score was >10. Patients with a CDAI of <150 or a CAI of <5 were considered to be in remission. We collected data from the files of all IBD patients including current medication, history of fistula, and primary sclerosing cholangitis (PSC).

Steroid refractory/dependent disease was defined following the recommendations of the European guidelines for CD and UC[12,13] as active disease despite prednisolone up to 0.75 mg/kg/day for a period of 4 weeks.[12,13] Steroid dependent disease was defined as the inability of reducing steroids below the equivalent of prednisolone 10 mg/day within 3 months of starting steroids without recurrent active disease or a relapse within 3 months after stopping the steroids.[12,13]

2.2. Urine samples

A 2 ml sample of the initial part of the first voided morning urine was obtained from every IBD patient and control. The subjects added 2 ml of this urine to a tube containing 8 ml of Carnoy solution (6/6/1 vol. ethanol/glacial acetic acid/chloroform), which was given to them by the investigator. The initial part of the urinary stream was used for the study to ensure that the concentration of epithelial cells was sufficient for analysis. The patients returned the samples to the outpatient clinic at their next appointment or delivered them directly to the Laboratory for Molecular Genetics, Polymicrobial Infections and Bacterial Biofilms in Berlin, Director: Dr. Alexander Swidsinski. The tubes were vortexed and then an aliquot of 1.5 ml was pipetted into an Eppendorf tube and then centrifuged for 6 min at 6000 *g*. The supernatant was removed from the sediment and 75 μl of Carnoy solution was added. The processed sample represented now is 300 μl of the initial urine sample and was stored in the Eppendorf tube at 4 °C until hybridization.

A 5 × 5 mm hybridization area was marked with a PAP Pen on a superfrost glass slide (Langenbrinck, Emmendingen, Germany). The sample was vortexed once more and a 5 μl aliquot (representing 15 μl of the initial sample) was pipetted within the area of hybridization. Four glass slides were prepared in this manner. The glass slides with the aliquots were dried for 30 min at 50 °C prior to hybridization.[6] In addition, three of the four glass slides of every sample were incubated with 20 μl of 1% lysozyme for 15 min at 37° C.

2.3. FISH

Five microliters of the hybridization solution was pipetted onto each glass slide. The slides were hybridized for 60 min at 50 °C. The protocol used was previously reported by Swidsinski et al.[6] The eight oligonucleotide probes listed in Table 1 were used.[8,14–20]

All glass slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Bacteria, epithelial cells and leukocytes were assessed in a multi-color analysis using a mix of three 16 or 23 rRNA probes on each of the four superfrost glass slides. Four combinations of oligonucleotide probes were used and are shown in Table 2.

Concentrations of epithelial cells and leukocytes were calculated per ml urine. The concentrations of the bacterial subgroups were estimated semiquantitatively in 5×10^5/ml steps by estimating the number of bacteria per field of view. This number was then used to approximate the concentration