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Positive effects of local therapy with a vaginal lactic acid gel on dysuria and *E.coli* bacteriuria question our current views on recurrent cystitis

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Abstract
Objective We tested the effect of vaginally applied lactic acid gel on symptoms and bacteriuria in acutely exacerbated recurrent *Eschericia coli* cystitis.
Methods Carnoy fixed samples of the morning urine from 20 women with a history of recurrent *E.coli* cystitis were prospectively investigated for bacteriuria using fluorescence in situ hybridization (FISH).
Results In 11/20 women with acute cystitis, the symptoms and bacteriuria were regressive with lactic acid gel treatment, without the need for antibiotic treatment. The complete regression of symptoms took between 1 week (7 women) and 4 weeks (4 women). In parallel with this regression, the microscopic shape of *E.coli* bacteria in these women changed from short rods to long curly filaments starting within the first days of therapy. The filamentous transformation affected 100% of the *E.coli* population in six women and at least 50% of *E.coli* population in five women and was not observed in urine samples from untreated women or in women without clinical response to lactic acid gel. This could not happen if the bladder was the origin of the infection.
Conclusions A number of recurrent and probably acute cystitis is a local vagino-urethritis caused by an adhesive invasive *E.coli* biofilm of the vaginal surface.

Keywords Repeat *E.coli* related cystitis · Bladder infections · Vaginal biofilms

Abbreviations

FISH	Fluorescence in situ hybridization
Cy3, FITC, Cy5, DAPI	Different fluorescent dyes corresponding to orange, green, dark red, and blue colors
ECO1167	*Eschericia coli*
Eub338	*Eubacteria* (virtually all bacteria)
BV	Bacterial vaginosis
Carnoy solution	A fixative with composition of 6/6/1 vol. ethanol/glacial acetic acid/chloroform

Introduction

Biofilms are crucial in heart valve endocarditis, prosthesis associated infections, bacterial vaginosis and recurrent cystitis. Different to planktonic growth, bacterial biofilms are highly resistant to host response, environmental stress and antibiotics [1]. Newer therapies seek to prevent their formation [1, 2]. Relactagel®, a lactic acid containing gel

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for personal vaginal hygiene, which can be purchased over the counter, was developed to mimic the pH and composition of healthy vaginal secretions, neutralize embarrassing odor and ease discomfort. Since some experiments indicated that lactic acid may inhibit *Gardnerella* biofilm in vitro [3], we tested whether similar effects can be observed in vivo. Bacterial vaginosis (BV) is a gynecological disorder, which is characterized by a prolific *Gardnerella* biofilm of the vaginal epithelium [4]. Urine sediments contain desquamated vaginal epithelial cells. Biofilm adhering to these can be assessed via fluorescence in situ hybridization (FISH). In a preliminary study, monitoring the *Gardnerella* biofilm in women with BV treated with vaginally applied lactic acid gel, one woman reported a marked relief of coincidental dysuric symptoms after starting treatment with vaginally applied lactic acid gel. To further investigate the effects of lactic acid gel on cystitis, we studied *E.coli* in urine samples using FISH and observed a marked change in the form of bacteria to long filaments, followed by a reduction and disappearance of *E.coli* after the lactic acid gel application.

Treatment of cystitis with antibiotics is often ineffective and associated with a high rate of recurrence. New therapeutic interventions are needed We therefore set out to test the effect of vaginally applied lactic acid on bacteriuria and *E.coli* morphology in urine samples using FISH.

Materials and methods

Subjects

1. Twenty women (age range 32–71 years) with a history of recurrent *E.coli* cystitis for at least 3 years and more than four episodes in the last 12 months, who were unresponsive to first line antibiotic treatment and who had multiple changes of antibiotics in the past. Nine of the women were postmenopausal. Seven had a hysterectomy. All women were previously seen by urologists or nephrologists, had normal renal function, no urological abnormalities and six of them had a prior normal cystoscopy. None of the women carried an intravaginal or uterine device. None of the women received hormone replacement.
2. The occurrence of adherent *E.coli* in the general female population was investigated in previously obtained vaginal biopsies from 160 women. The vaginal biopsies were fixed in Carnoy solution and investigated for adherent biofilms previously [4]. The biopsies were from women with bacterial vaginosis and controls. The women with positive findings were contacted and questioned for a history of cystitis complaints.
3. Two thousand and five hundred urine samples from different untreated patients collected in the last 2 years were investigated for the occurrence of "spontaneous" filamentous transformation of *E.coli*.

Study design

Two microliters of spontaneously voided morning urine was fixed by adding 8 ml of Carnoy solution. The fixing was performed by the patient immediately after obtaining the morning urine.

The urine samples were collected every 2–4 weeks as long as the women were free of complaints. At the time of relapse of cystitis, the women were instructed to collect urine samples daily and visit the clinic of the Charité hospital at their earliest convenience. At this visit, the complaints were recorded, women were provided with lactic acid gel (Relactagel®). Seven women, who lived a far distance from the hospital, received the gel ahead of the anticipated event. We recommended that the gel be applied vaginally in the evening before going to bed daily for 4 weeks. Thus the local gel therapy started between the 2nd and 6th day after symptoms began. It was planned that the urine samples during therapy were collected on the first day of the treatment and then repeated every 2 days on Monday, Wednesday and Friday. In case of a positive effect, this mode of collection was continued for the duration of 4 weeks. In case of aggravation of symptoms, the treatment was stopped immediately. If the complaints persisted, the application of the gel and the urine sample collection were terminated and antibiotic treatment was started.

Preparation of the urine samples

An aliquot of 2 ml Carnoy fixed urine (2 ml of urine mixed with 8 ml of Carnoy fixative) was centrifuged for 6 min at 9,000*g*. The sediment was decanted and 1 ml of Carnoy solution was added and left at room temperature. After 1–5 min, the sediment was centrifuged once more (6 min/9,000 G), decanted, 50 µl Carnoy solution was added and then stored at 4°C until hybridization. If more hybridizations were planned, larger urine aliquots were prepared in the same manner as described above.

A 5 × 5 mm quadrant area of hybridization was marked with a PAP Pen on a superfrost plus glass slide. The Carnoy fixed urine sediment was vortexed, 5 µl aliquots were pipetted within the area of hybridization and dried for 30 min at 50°C just prior to the hybridization.

Five microliters of the final aliquot was used for single hybridizations and represented 20 µl of the initial urine volume.

FISH

Bacteria were assessed in a multi-color analysis using a mix of two probes: ECO1167-Cy3[5], Eub338-FITC and DAPI counter stain according to previously described protocols [4, 6] using a Nikon e600 fluorescence microscope, Nikon DXM1200F camera and accompanying software (Nikon, Tokyo, Japan). Other bacterial groups embedded within the biofilm were assessed using group-specific probes as described [4].

Bacterial enumeration

Bacteria were enumerated within the whole area of hybridization when low concentrations were present or within an area of 100 × 100 μm when high bacterial concentrations were present and multiplied with 50 or 1,250, respectively.

Bacterial culture

Because of the Carnoy fixing, the bacterial culture of the uropathogenic strains could not be performed in all women. *E.coli* strains were isolated from two women with clinical response to test the influence of the lactic acid gel on *E.coli* growth in vitro. *E.coli* strains were plated on MacConkey agar. A gel was poured across half of the plate. The plate was then incubated at 37°C overnight. Colonies grown with or without contact with the gel were fixed in Carnoy and investigated using FISH for their morphology.

Results

All 20 women experienced an episode of acute cystitis within 6 month after inclusion into the study and applied lactic acid gel initially. The intended number and intervals for the sample collections were fulfilled as follows: in all women at least two samples of morning urine were obtained prior to a relapse of acute cystitis and one to two samples of urine were collected during symptoms of acute cystitis prior to therapy. During gel application, the urine samples were collected in all women at least two times a week and in six women three times per week. One to three monthly urine samples were collected in 12 women after the 4 weeks of the gel treatment.

Clinical effects

Five women reported no improvement, two women reported an aggravation of the dysuria and burning pains in the vagina and two reported initially a minimal improvement, with no improvement over time, the symptoms remained severe. These nine women with negative, lacking or inadequate response were subsequently treated with antibiotics.

The application of the gel was stopped when antibiotic treatment was started (days 3–9).

Eleven women reported significant improvement of their complaints starting with the first gel application. Treatment with the gel in these women was continued. In seven, the clinical symptoms disappeared completely within 1 week. The total disappearance of symptoms took 4 weeks in the other four women. Five of these 11 women continued with *E.coli* bacteria in their urine in concentrations of $<10^3$/ml, but were asymptomatic.

None of the 11 women with response to lactic acid gel required antibiotic treatment in the following 2 months. Four women had no relapse within 3 months of follow-up.

Seven women were observed further for 4–12 months. Three of these seven women refused to stop the gel application after the study. Relapse of dysuria occurred in four women. The continuation of the daily application did not prevent the relapse of cystitis (5 and 9 months later) in two of the three women who continued with the gel.

Effects on bacteriuria

During the asymptomatic period, 2–6 samples from each woman were obtained, resulting in a total of 66 samples. *E.coli* was not detected in 49 samples (76% of samples) from 11 women (55%) and was found in low concentrations ($<10^3$ bacteria/ml) in 13 samples from 7 women. *E.coli* of 10^4/ml was documented in four of nine samples from two asymptomatic women. Concentrations of 10^5/ml or higher were never observed.

In the symptomatic period prior to treatment, 31 urines were collected. All samples contained bacteria in concentrations of $>10^4$/ml starting with day 1 of symptoms.

E.coli was predominant in 18 of 20 women with concentrations between 10^4 and 10^6/ml. The fluctuations in concentrations between single samples from the same women taken on consecutive days could vary but never fell below 10^4/ml. Concentrations of 10^6/ml were observed in three samples. *E.coli* had a coccoid short rod form (Fig. 1).

A mixed bacteriuria was observed with *Enterococcus* as a predominant bacterium in two women. No *E.coli* was detected in one and the *E.coli* concentration was below 10^3/ml in the other woman.

During lactic acid gel application, the bacteriuria remained high $>10^4$/ml in all samples of the nine women without or with insufficient clinical response. No changes in the form of *E.coli* bacteria were observed in women without clinical response.

A gradual decrease in *E.coli* concentrations was observed in the 11 women with clinical response, see Table 1. This decrease was relatively slow and reached the

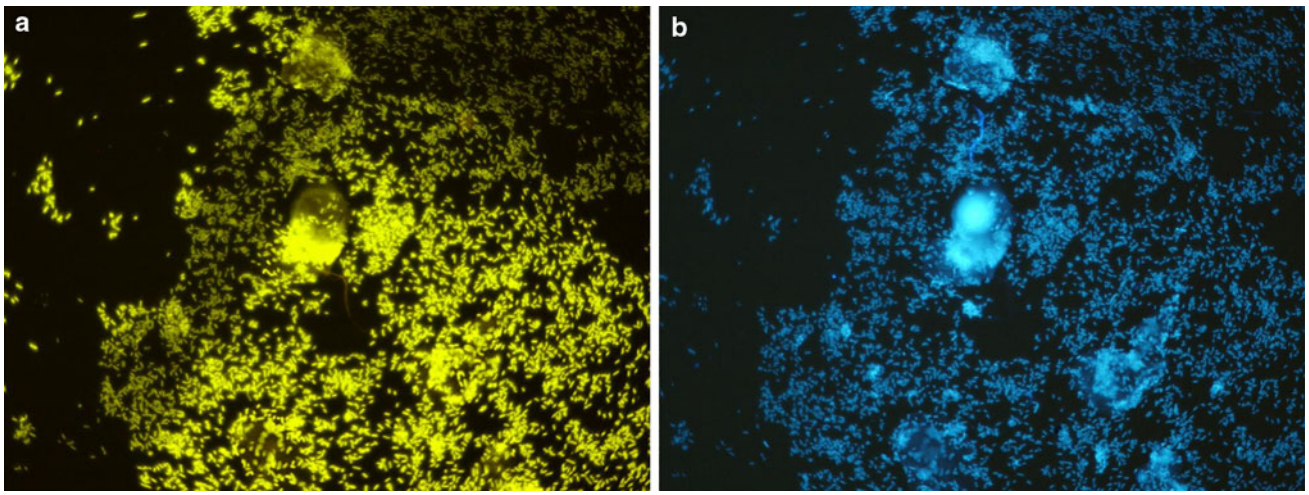

Fig. 1 52-year old woman with 2 days of acute cystitis. **a** *E.coli* bacteriuria in Carnoy fixated sample of morning urine from a woman with acute exacerbation of recurrent cystitis hybridized with ECO1167-Cy3 probe (*orange fluorescence* ×400). *E.coli* bacteria have the form of short "coccoid" rods. **b** The same microscopic field stained with DAPI, a stain that visualizes nucleic acids (*blue fluorescence*). Remnants of desquamated epithelial cells are clearly seen here. Comparing figure one and two, one can see that *E.coli* cells are partially adherent to remnants of epithelial cells.

Table 1 *E.coli* occurrence and concentrations in urine samples of women with response to lactic acid gel treatment ($n = 11$)

Pretreatment		Lactic acid gel treatment			
	Day −2 to 0	Day 1 to 7	Day 8 to 14	Day 15 to 21	Day 22 to 28
Mean *E.coli* concentrations $\times 10^5 \pm$ SD/ml	2.1 ± 2.3	1.7 ± 2.6	0.5 ± 1.8	0.01 ± 0.04	0.003 ± 0.007
Occurence in samples	100% (19/19)	100% (31/31)	94% (30/32)	55% (17/31)	27% (8/30)
Occurence in patients	100% (11/11)	100%	100%	91% (10/11)	45% (5/11)
P -compared to pre-treatment period		ns	0.01	<0.001	<0.001

level of statistical significance only in the second week of treatment ($p = 0.01$). The difference in *E.coli* concentration was highly significant, starting at week 3 (Table 1). During the 4th week, 6 of the women had no *E.coli* in their urine samples. In 5 of the 11 women, *E.coli* bacteriuria of $<10^3$/ml was observed. In the 11 women with clinical response, the morphology of *E.coli* bacteria changed dramatically starting with the second day of the gel application. In seven women, the *E.coli* bacteria that initially had a short coccoid rod form, changed their form to long filaments of up to 40 µm length and were partially clewed (Fig. 2a). In four women, the filaments were lacking, but a marked elongation of the *E.coli* to "long rods" was observed.

In six women the total population of *E.coli* bacteria seen under the microscope was involved in the filamentous transformation. In five women between 50 and 90% of the *E.coli* population was transformed. Over the time of the gel application the elongation of the *E.coli* form remained constant. After the end of the gel application, when bacteria were still detectable in low concentrations, the form of the *E.coli* changed back to short coccoid rods.

In vitro, the gel did not suppress the growth of *E.coli* visually. FISH of the bacteria taken from *E.coli* colonies growing while covered by the gel, however, showed a changed morphology. Five percent of *E.coli* bacteria growing on MacConkey and covered with the gel had a long rod form (Fig. 2b).

Vaginal biopsies from the general population

One hundred and thirty-eight of 160 vaginal biopsies had no *E.coli*. A single signal, with a form conform with *E.coli* bacterium, was observed in the proximity of the vaginal epithelium in 20 women. We regarded this as a contamination or bias. In two of 160 women a clear coat of adherent *E.coli* covered the epithelial surface of the biopsy (Figs. 3, 4). The concentrations of adherent *E.coli* to the vaginal epithelium were high and reached 6×10^5 bacteria/ml in one woman and 2×10^6 in the other. Both women with an adherent *E.coli* coat complained of vaginal discomfort, itching and pain after intercourse. One of the women had a history of recurrent cystitis.

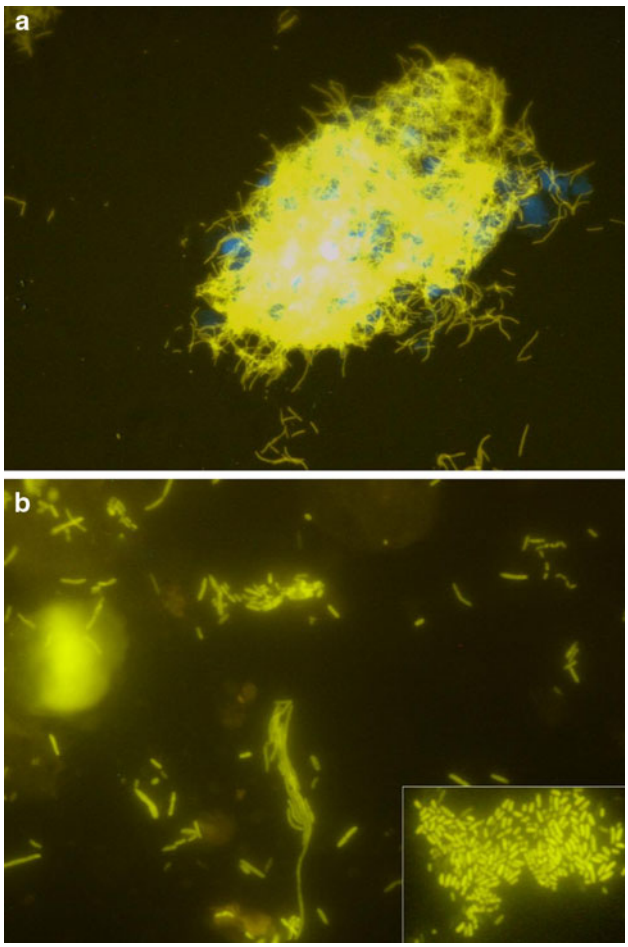

Fig. 2 **a** The same women as presented in Fig. 1: Acute *E.coli* cystitis, day 4 of the locally applied lactic acid gel. The positive hybridization signals with *E.coli* specific Cy3 stained FISH probe (*yellow fluorescence*) and DAPI stain (*blue fluorescence* ×400) are overlaid. One can clearly see the transformation of *E.coli* from coccoid (Fig. 1) to long "fusobacteria-like" filaments, which are organized as balls of threads enwrapping remnants of epithelial cells. **b** *E.coli* strain isolated from the same women as in Fig. 2a. Bacteria are grown aerobically on MacConkey agar and covered with lactic acid gel. Despite visually unimpaired growth, part of the bacteria changed their form to long rods (×400)

E.coli in urine of untreated subjects

Using FISH, *E.coli* was detected in 820 samples of 2,500 investigated samples from untreated subjects and patients with urinary infections. A filamentous transformation of *E.coli* was never observed.

Discussion

Our study demonstrates positive clinical and bacteriostatic effects of vaginally applied lactic acid gel in 55% of women with exacerbation of recurrent *E.coli* cystitis.

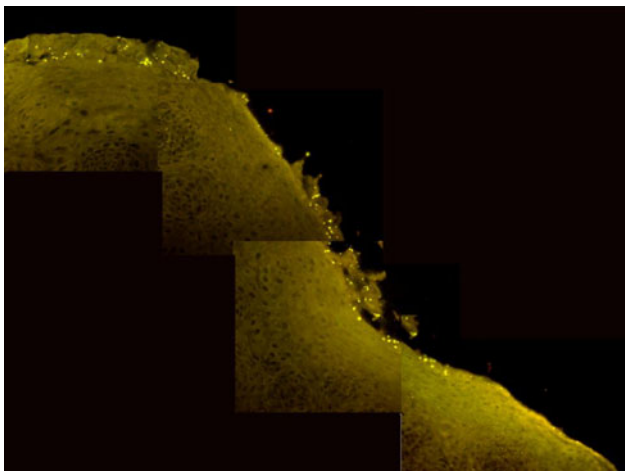

Fig. 3 Vaginal biopsy hybridized with the ECO1167-Cy3 probe (*yellow fluorescence* ×400). The figure is composed of 4 microscopic fields taken consecutively along the biopsy surface. *E.coli* biofilm is covering the whole biopsy surface

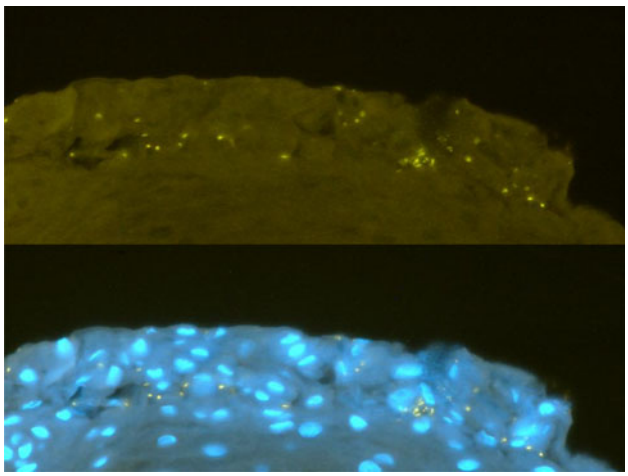

Fig. 4 One of the microscopic fields from Fig. 3 hybridized with *E.coli* specific FISH probe (*above*) and counter stained with DAPI (*below*). One can clearly see the invasive location of *E.coli* bacteria within the epithelial layer in this part of the biopsy

The study was not double blind controlled and can not delineate the exact effect of the therapy on symptom resolution or *E.coli* clearance. The immediate symptomatic improvement could be, for example, due to soothing of burning pains in the uro-vaginal area. All women with clinical response to lactic acid gel therapy insisted that the last relapses of cystitis preceding the study had not spontaneously regressed and needed antibiotics therapy. Such statements in the absence of placebo controls may be highly biased and subjective. However, the filamentous transformation of bacteria was observed exclusively in women with a response to intravaginal lactic acid gel therapy and not in women without clinical and bacteriologic improvement. The filamentous transformation of

E.coli was also absent in all 850 urine samples positive for *E.coli*, randomly collected from untreated subjects. This indicates that the link between filamentous transformation, bacteriuria clearance and intravaginal lactic acid gel application is not accidental. We can only speculate about mechanisms behind this link.

The filamentous transformation of *E.coli* was first described as an effect of beta-lactam antibiotics that prevented the formation of a bacterial wall. Bacteria fail to disconnect from each other after their division and grow for a while as long filaments. [6, 7] The lactic acid gel contains no antibiotics. The lactic acid that regulates the low pH of the gel, can interfere with bacterial growth directly, and *E.coli* strains isolated from women with positive response changed their morphology while cultured on MacConkey agar in contact with lactic acid gel. But the changes in the culture were not as impressive as in the native urine of the treated women. The locally applied lactic acid gel can also disturb the *E.coli* life cycle indirectly through changes of the vaginal environment [8], local innate immunity [9] or shift in the composition of the vaginal microbiota [10, 11]. Filamentous transformation of *E.coli* was also reported as part of the life cycle of uropathogenic *E.coli* to subvert innate defenses of the host [12]. We do not know, what the transformation of *E.coli* in our patients means in terms of pathogenicity, but it is an extremely notable feature. Presently, we have investigated more than 4,000 urine samples from different women using FISH on Carnoy fixated sediments and never observed the transformation that we have observed in this study and in some cases after antibiotic treatment.

Whatever the mode of action of lactic acid on *E.coli* morphology or reasons of the transformation may be, the fact that the locally applied gel affects all of the *E.coli* in urine samples of six of the women is most remarkable. How can a vaginally applied substance affect bacteria, whose origin is thought to be in the bladder or higher up? Since urine was fixated, post voiding effects can be excluded. It is also unlikely that the gel could reach the urinary bladder. The only reasonable explanation seems to be that a subset of clinically diagnosed cystitis is in reality a local vagino-urethral infection. Indeed, how do we know that "cystitis" takes place in the urinary bladder? The diagnosis is based on dysuria paired with bacteriuria. Since bacteria are found in the urine, we assumed that their origin must be from the urinary tract. However, how valid is this assumption? The cystoscopy perfomed in women with recurrent cystitis reveals no pathology in most cases. Dysuria and bacteriuria may both originate from the vaginal-urethral region. The well-known phenomenon of honeymoon cystitis, cystitis after a change of partner and quick intercourse [13–15] are much more in accordance with a vagino-urethral origin of complaints, than with the urinary bladder as the place of infection. The vagino-urethral origin of recurrent cystitis could also explain why a seemingly simple disease proves to be extremely recalcitrant and difficult to treat in some cases. The systemic application of renal secreted antibiotics may be insufficient to reach the population of the vaginal *E.coli* biofilm. We do not feel that the lactic acid gel treatment is the perfect solution. Continuing the application of the gel did not prevent the relapse of "cystitis" in two of three women, who continued the gel application after the end of the study. However, lactic acid gel is the first substance of the art, which may help in the development of more effective treatment in the future. Presently, no vaginal antibiotic is available, to which *E. coli* is primarily susceptible. *E.coli* is not even seen as a component of the vaginal microbiota. Its occasional appearance here is explained by the anatomically close perianal region. To estimate the occurrence of adherent vaginal *E.coli* biofilms in the general population, we reinvestigated 160 Carnoy fixated vaginal biopsies from earlier studies including women with BV and controls [5]. *E.coli* diffusely adhered to the vaginal epithelium in concentrations of $10^{5–6}$ bacteria/ml in two of 160 vaginal biopsies. Both of the women had local inflammation of the vagina with local itching and burning pains. One of the women had in addition a history of recurrent cystitis.

In summary, we agree with the previous suggestions that the *E.coli* bacteriuria may often not be due to infection of the urinary bladder, but due to vagino-urethritis. However previously, the vaginourethritis was discussed mainly in terms of ascending infection leading to cystitis and, therefore, local estriol treatment or urethroplasty surgery was introduced [14, 15]. Our present data, however, indicate that in some patients the vagino-urethritis symptoms may be mimicked by an adherent *E.coli* biofilm without involvement of the urine bladder.

Prospective studies using local intra-vaginal antibacterial therapy should also be considered in cases where the origin of the bacteria is of vagino-urethral origin.

The study was approved by the Ethics Committee of the Charité Hospital.

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Conflict of interest We declare that we have no conflict of interest.

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