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Role of Urinary Cathelicidin LL-37 and Human β-Defensin 1 in Uncomplicated *Escherichia coli* Urinary Tract Infections

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Cathelicidin (LL-37) and human β-defensin 1 (hBD-1) are important components of the innate defense in the urinary tract. The aim of this study was to characterize whether these peptides are important for developing uncomplicated *Escherichia coli* urinary tract infections (UTIs). This was investigated by comparing urinary peptide levels of UTI patients during and after infection to those of controls, as well as characterizing the fecal flora of participants with respect to susceptibility to LL-37 and *in vivo* virulence. Forty-seven UTI patients and 50 controls who had never had a UTI were included. Participants were otherwise healthy, premenopausal, adult women. LL-37 MIC levels were compared for fecal *E. coli* clones from patients and controls and were also compared based on phylotypes (A, B1, B2, and D). *In vivo* virulence was investigated in the murine UTI model by use of selected fecal isolates from patients and controls. On average, UTI patients had significantly more LL-37 in urine during infection than postinfection, and patient LL-37 levels postinfection were significantly lower than those of controls. hBD-1 showed similar urine levels for UTI patients and controls. Fecal *E. coli* isolates from controls had higher LL-37 susceptibility than fecal and UTI *E. coli* isolates from UTI patients. *In vivo* studies showed a high level of virulence of fecal *E. coli* isolates from both patients and controls and showed no difference in virulence correlated with the LL-37 MIC level. The results indicate that the concentration of LL-37 in the urinary tract and low susceptibility to LL-37 may increase the likelihood of UTI in a complex interplay between host and pathogen attributes.

Urinary tract infection (UTI) is one of the most common infections, with 60% of all women suffering from a UTI at least once in their lifetime (1). The major pathogen is *Escherichia coli*, which is responsible for 80% of all UTIs in uncompromised patients (2). In the majority of cases, bacterial pathogens causing UTIs in otherwise healthy individuals originate from the fecal flora, with the vagina as a possible reservoir on the way to the urethra and, finally, the bladder (3).

The urinary tract is lined with numerous defense mechanisms in order to prevent infections, and antimicrobial peptides (AMPs) play an important role in this defense. The uroepithelium expresses AMPs, e.g., α-defensin 5, RNase 7, β-defensin 1 (hBD-1), and cathelicidin (LL-37) (4–7), among which hBD-1 and LL-37 are the focus of this study.

LL-37 is constitutively expressed at low levels in urothelial cells, renal epithelial cells, and circulating cells, e.g., neutrophils, in which the peptide is stored in granules (7, 8). Neutrophil release of the peptide ensures a rapid increase in the local concentration upon bacterial presence until epithelial production is increased for further protection (9). Expression can be increased by vitamin D (10). It is known that *Camp*$^{-/-}$ mice, lacking mCRAMP, the mouse homologue of human LL-37, have more *E. coli* bacteria attached to the bladder than wild-type mice, indicating an important role of this peptide in protection against urinary tract infections (7). Chromek et al. (7) showed that human (LL-37) and murine (mCRAMP) cathelicidins play an important role during bladder infection and provided evidence that cathelicidin expression and secretion were increased during *E. coli* urinary tract colonization. Children with cystitis or pyelonephritis had increased concentrations of LL-37 in the urine compared to children without infection (7). A similar study has not been performed on adults or on patients with uncomplicated urinary tract infection, such as cystitis. It is therefore not known at this point whether pyelonephritis, cystitis, or both in adults upregulate LL-37 production. Finally, levels of LL-37 have not been determined for UTI patients postinfection for comparison with levels in controls.

hBD-1 is expressed in epithelial cells, with the highest concentrations in the kidneys and the female reproductive tract. hBD-1 also serves a role as a chemoattractant for immature dendritic cells. Like LL-37, hBD-1 is constitutively expressed and is known to be upregulated approximately three times during pyelonephritis (12). *Defb1*$^{-/-}$ mice lacking β-defensin 1 have 30% more bacteria in their urine than wild-type mice, indicating that hBD-1 plays a role in developing urinary tract infections (13). Furthermore, three single nucleotide polymorphisms (SNPs) in the 5′-untranslated region of *Defb1*, namely, G-20A, C-44G, and G-52A, have been correlated with host susceptibility toward, e.g., *Pseudomonas aeruginosa* in cystic fibrosis patients (14), HIV infection (15, 16), Crohn's disease (17), sepsis (18), and asthma (19). The role of these SNPs is not fully understood, but it is hypothesized that they influence peptide production or function (14, 20). It has not been investigated whether these SNPs have a correlation with UTI.

LL-37 and hBD-1 both have a direct killing mechanism toward *E. coli*. Only a few studies have been performed in an attempt to describe the susceptibility of *E. coli* to these peptides, and these are

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based on few *E. coli* isolates (21–24). It has been shown that hBD-1 has antimicrobial effects toward *E. coli* regardless of whether it is denatured or not (25). Additionally, it has been shown that bacteria causing pyelonephritis are more resistant to LL-37 than bacteria causing cystitis (7). If hBD-1 and LL-37 prove to be significant for developing UTI, it would be relevant to determine the susceptibility to these peptides of fecal *E. coli* isolates from UTI patients and controls.

The aim of this study was to identify the role of LL-37 and hBD-1 in the development of lower urinary tract infections. This was performed by comparing women with uncomplicated UTIs to female control subjects who had never had a UTI.

MATERIALS AND METHODS

Study participants. This study was approved by The Danish National Committee on Biomedical Research Ethics and the Danish Data Protection Agency. Study participants were recruited prospectively in a case-control study from two general practices in Denmark, serving approximately 14,000 and 13,000 inhabitants. Inclusion criteria for UTI patients were as follows: otherwise healthy, nonrelated, premenopausal women presenting $\geq 10^4$ CFU of *E. coli* and leukocyturia as well as symptoms of lower UTI. Inclusion criteria for controls were as follows: healthy, nonrelated, premenopausal women who had never had a UTI, presenting a urine sample without bacteria or leukocyturia. Exclusion criteria for both patients and controls were as follows: postmenopausal status, diabetic, pregnant, close relation to another study participant, and recent surgery in the urogenital area.

Fifty cases and 53 controls were included in the study. Epidemiological data were collected from the medical journals of the patients, including age, overall general health, number of UTI infections, previous antibiotic treatment, and other diseases. Recurrent infection was defined as an episode with two samples with $\geq 10^4$ bacteria, leukocyturia, and symptoms of UTI within 30 days of a previous episode.

Samples. UTI patients delivered a urine sample and a rectal swab at presentation to their doctor. Subsequently, each patient delivered a urine sample again >1 week after termination of antibiotic treatment. If the second urine sample contained bacteria and/or leukocytes, a third sample was required >1 week after finishing antibiotic treatment for a reinfection or, in cases where a reinfection was not the case, >2 days after the last sampling. Control individuals delivered a urine sample and a rectal swab at the time of inclusion. Urine samples were analyzed by use of urine dipsticks, microscopy, and quantitative culture of urine.

All urine samples were stored at 5°C until frozen. Samples were centrifuged at 2,000 × *g* for 10 min, aliquoted into portions of 250 μl in Eppendorf tubes, and frozen at −80°C no later than 48 h after sampling. Fecal swabs (Copan E-swab; Copan Diagnostics Inc.), were collected by the study participants themselves, with a printed guideline as support.

Bacterial isolates. Each rectal swab was plated within 48 h on chromogenic agar plates, on which *E. coli* can be distinguished from other species by its pink color. From each plate, up to 20 *E. coli* colonies were tested on *p*-nitrophenyl-β-D-glucopyranosiduronic acid (PGUA) agar plates to confirm the identity of *E. coli* by its β-glucuronidase activity. In case of discrepancies, additional plating was performed on bromthymol blue-sucrose agar (BS agar) and followed by testing using the API-20E test (bioMérieux). Isolates were frozen and kept at −80°C.

UTI isolates were isolated from Flexicult agar plates (SSI Diagnostica), the diagnostic procedure used by the general practitioners, and confirmed as *E. coli* as described above before being frozen in glycerol vials at −80°C.

RAPD PCR. All isolates were typed by random amplified polymorphic DNA by PCR (RAPD PCR) in order to identify the number of clones in each sample and whether the infecting UTI isolate was found in the fecal flora. A RAPD PCR assay was developed for this study. In short, two PCRs, each using one primer, i.e., 1247 (AAGAGCCCGT) (26) or 1283 (GCGATCCCCA) (27) (Tag Copenhagen), were run on all fecal isolates. Multiplex master mix without Q solution (Qiagen) was used as recommended by the manufacturer, and each 25-μl reaction mixture contained a 2 μM concentration of one of the two primers and 2.5 μl of template DNA. Cycling conditions for 1247 PCR were as follows: 95°C for 15 min followed by 35 cycles of 94°C for 1 min, 38°C for 1 min, and 72°C for 2 min, with a final step of 72°C for 10 min. Cycling conditions for 1283 PCR were as follows: 95°C for 15 min followed by 35 cycles of 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min, with a final step of 72°C for 10 min. Template DNA was prepared by adding one loopful of bacteria (1-μl inoculation needle) from an overnight agar plate to 300 μl of DNase-free water, followed by incubation at 95°C for 10 min. All isolates from the same person were included in the same PCR run and gel. This was also the case for comparing a UTI isolate from a patient with its respective fecal clones. The criterion for distinguishing clones was >1 band difference in at least one of the PCRs.

Phylotyping. Phylotyping was performed on unique clones based on RAPD typing by multiplex PCR, which was modified from the work of Clermont et al. (28). Briefly, each 25-μl reaction mixture contained multiplex master mix and Q solution (Qiagen) according to the recommendations of the manufacturer, 0.2 μM (each) primers, and 1 μl of template DNA. Cycling conditions were as follows: 15 min at 95°C followed by 29 cycles of 30 s at 94°C, 90 s at 58°C, and 60 s at 72°C, with a final step of 10 min at 72°C.

As recommended by Gordon et al. (29), an additional PCR for *ibeA* was performed on isolates positive for *chuA* and Tspe4.c2. *ibeA* PCR was performed with the primers 5′-TGGAACCCGCTCGTAATATAC-3′ (forward) and 5′-CTGCCTGTTCAAGCATTGCA-3′ (reverse) (Tag Copenhagen), as follows. Multiplex master mix (Qiagen) with Q solution was used as recommended by the manufacturer, and each 25-μl reaction mixture contained 0.4 μM (each) primers and 1 μl template DNA. Cycling conditions were 15 min at 95°C followed by 29 cycles of 30 s at 94°C, 30 s at 56°C, and 60 s at 72°C, with a final step of 10 min at 72°C. Phylotypes (A, B1, B2, D, and NT [nontypeable]) were evaluated as recommended by Gordon et al. (29).

DNA extraction and SNP analyses. Human DNAs from study participants were purified using a urine (exfoliated cell) DNA isolation kit (Norgen Biotek) directly on pellets formed from centrifugation of urine samples at 650 × *g*. The gene encoding hBD-1 consists of two exons separated by one intron. Exon 1 encodes the signaling peptide for hBD-1, and exon 2 encodes the actual peptide. Primers (Tag Copenhagen) were designed for amplification of the two exons. For exon 1, the forward primer 5′-CTCCCTTCAGTTCCGT-3′ and reverse primer 5′-CTTGTTCCTCGTCCCTT-3′ were applied. For exon 2, the forward primer 5′-AAACAAAGTGGAGGTGACAT-3′ and reverse primer 5′-TCCGGTGTTTTGCAGTTATT-3′ were applied. For both exons, each 25-μl reaction mixture contained multiplex master mix (Qiagen) with Q solution as recommended by the manufacturer, along with 0.4 μM (each) primers. Cycling conditions for exon 1 were 15 min at 95°C followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with a final step of 10 min at 72°C. Cycling conditions for exon 2 were 15 min at 95°C followed by 40 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 1 min, with a final step of 10 min at 72°C. SNPs were identified by alignment, using CLC Main Workbench software (CLC Bio).

Contents of antimicrobial peptides in urine. hBD-1 concentrations were measured by an enzyme-linked immunosorbent assay (ELISA) specific for hBD-1 (Alpha Diagnostic). LL-37 concentrations in urine were measured using a human LL-37 ELISA (Hycult Biotech) after optimization and validation for use in urine. The kit was optimized and validated to measure concentrations of LL-37 in urine over the range of 0.06 to 11 μg/liter. The assay exhibited parallelism of results between serial dilutions. The detection limit was 0.06 μg/liter, and the intra-assay and inter-assay variabilities were 6.8% (range, 0.74 to 28.2%) and 12.9% (range, 5.25 to 19.5%), respectively. Samples were incubated in 96-well microtiter plates for 4 h instead of the 1 h prescribed in the kit protocol. All runs were performed with sample and kit controls in both ends of the plate.

Levels of hBD-1 and LL-37 were normalized to levels of urinary creatinine in samples and hence were expressed as amounts of antimicrobial peptide/g creatinine. Creatinine concentrations were determined using the Microvue creatinine assay (Quidel, Electra-Box Diagnostics).

Susceptibility of *E. coli* to LL-37. Susceptibility to LL-37 was tested on all UTI isolates, fecal clones from controls, and fecal-only clones (fecal clones different from the infecting UTI clone) from patients, all in two different broth dilution assays, one with biofilm inhibition and one that was biofilm promoting, as inspired by Kai-Larsen et al. (24). LL-37 (Bachem) was of >95% purity and was reconstituted in 0.01% acetic acid, aliquoted, and stored at −80°C until used. Both assays were performed in 96-well polypropylene plates. LL-37 fractions were thawed only once, and 2-fold dilutions with 0.01% acetic acid were prepared. The microtiter plate contained 11 dilutions of peptide (concentrations of 0.01 to 12.5 mg/liter) and a blank containing 0.01% acetic acid. *E. coli* isolates were plated on 5% blood agar plates, grown overnight, and suspended in a modified Trypticase soy broth (TSB) (10% TSB supplemented with salt, as described by Turner et al. [30]) to an optical density (OD) of 0.12 to 0.13, equivalent to a 0.5 McFarland standard and 10^8 CFU/ml. This suspension was diluted 100-fold, to a concentration of 10^6 CFU/ml. The final concentration of *E. coli* in the plate was 4.5×10^5 CFU/ml. For each run, *E. coli* ATCC 25922 was included as a quality control. Isolates with MICs of >12.5 mg/liter were rerun with concentrations of up to 100 mg/liter.

The biofilm-promoting assay was performed similarly to the above-described assay, but with the following modifications. Initial plating of bacteria was performed on LB agar without salt in order to promote biofilm growth, as described elsewhere (24). Broth for this assay was identical to the above-described broth, but without added salt for biofilm induction. In this assay, peptide concentrations were in the range of 0.01 to 100 mg/liter.

Urinary tract infection mouse model. Twelve isolates were selected for virulence testing in a urinary tract infection mouse model that is described elsewhere (31). The experiments were permitted by the Animal Experiments Inspectorate under the Danish Ministry of Food, Agriculture and Fisheries. Investigated isolates included fecal isolates from controls and UTI patients, having phylotypes B2 and D ($n = 3$ for each phylotype) and exhibiting a low biofilm LL-37 MIC of 12.5 mg/liter, as well as fecal isolates having phylotypes B2 and D ($n = 3$ for each phylotype) and exhibiting high biofilm LL-37 MICs of ≥100 mg/liter. Additionally, a positive-control isolate (*E. coli* C175-94; Statens Serum Institut) was included as a quality control.

The procedure was performed as previously described by Jakobsen et al. (32). Briefly, mouse bladders were emptied prior to transurethral inoculation with 50 μl of bacterial suspension (10^9 CFU/ml). Each *E. coli* isolate was inoculated into five (all isolates but one phylotype B2/high LL-37 MIC isolate and two phylotype D/high LL-37 MIC isolates) or six (one phylotype B2/high LL-37 MIC isolate and two phylotype D/high LL-37 MIC isolates) outbred female albino OF1 mice (28 to 32 g; Charles River Laboratories). Urine was collected from the mice at 72 h postinoculation, and mice were euthanized by cervical dislocation, followed by removal of the bladder and kidneys. Urine samples were processed on the same day, whereas kidneys and bladders were stored in 0.9% saline solution at −80°C until processed. Bacterial counts were determined for the urine, bladder, and kidneys. The detection limit was 25 CFU/ml.

Statistics. GraphPad Prism 5 and GraphPad InStat 3 (GraphPad Software) were used for all statistical analyses, with the exception of regression analyses and Tobit modeling, which were performed with SAS 9.3 (SAS Institute).

LL-37 concentrations in urine were log transformed for normalization. Log-transformed concentrations of LL-37 in urine were evaluated by Tobit modeling on a normal scale in order to take missing data into account. We also applied Tobit modeling in order to correlate the concentration of LL-37 to the number of CFU/ml in urine. Statistical analysis of hBD-1 was performed by analysis of variance (ANOVA) followed by two-tailed unpaired *t* tests applying the Welch and Bonferroni correction ($\alpha = 0.025$).

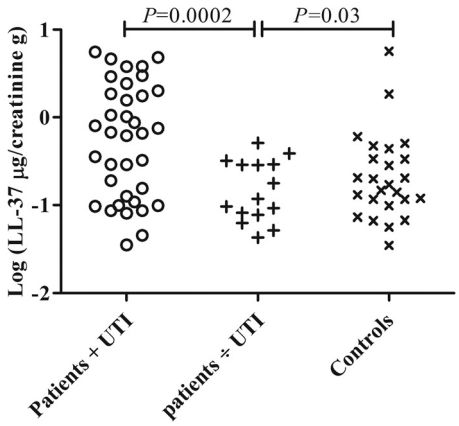

FIG 1 Log concentrations of LL-37 normalized to creatinine concentrations in urine samples from UTI patients during infection (patients + UTI) and after infection (patients ÷ UTI) and from controls. The level during infection was higher than that after infection ($P = 0.0002$ by Tobit modeling), and baseline levels for patients were lower than those for controls ($P = 0.03$ by Tobit modeling). Missing data were not plotted.

LL-37 MICs were compared by Fisher's exact test, with the Bonferroni correction in case of multiple comparisons. Results from the UTI mouse model were evaluated by the Kruskal-Wallis test to compare CFU counts of all isolates in the urine, bladder, and kidneys ($\alpha = 0.05$).

For SNP analysis, the χ^2 test for independence was performed ($\alpha = 0.05$) to analyze whether the distribution of the wild-type, heterozygous, and homozygous forms of the three SNPs was independent of being a UTI patient or control. Additionally, logistic regression was performed in order to analyze whether patients with recurrence had a specific combination of the three SNPs.

Regression analyses were performed on UTI patients in order to analyze whether concentrations of LL-37 and hBD-1 during and after infection were correlated with the total number of UTIs and recurrence.

RESULTS

Study participants. Three controls and three patients were excluded for the following reasons: two controls proved to be close relatives, and hence the second individual was excluded; according to their medical journals, two controls had had a UTI; two patients were postmenopausal according to their medical journals; and one patient was recruited but proved to have only 10^3 *E. coli* organisms/ml. The final numbers of patients and controls included in the study were 47 and 50, respectively. The mean ages of patients and controls did not differ significantly (averages ± standard deviations [SD] of 35.2 ± 7.3 and 37.4 ± 8.5 years, respectively; $P = 0.2$ by the unpaired *t* test). Sixteen patients (34%) developed recurring infections.

Concentration of LL-37 in urine. Concentrations of LL-37 were determined for urine samples from UTI patients during and after infection, as well as for urine samples from healthy controls who had never had a UTI (Fig. 1). The concentration of LL-37 in urine was significantly higher during infection than after infection for all UTI patients but one (Fig. 1) ($P = 0.0002$ by Tobit modeling). Additionally, LL-37 baseline levels of controls were significantly higher than the levels for UTI patients after infection (Fig. 1) ($P = 0.03$ by Tobit modeling).

UTI patient LL-37 levels postinfection were low and did not enable measurement of LL-37 in all samples obtained. For samples

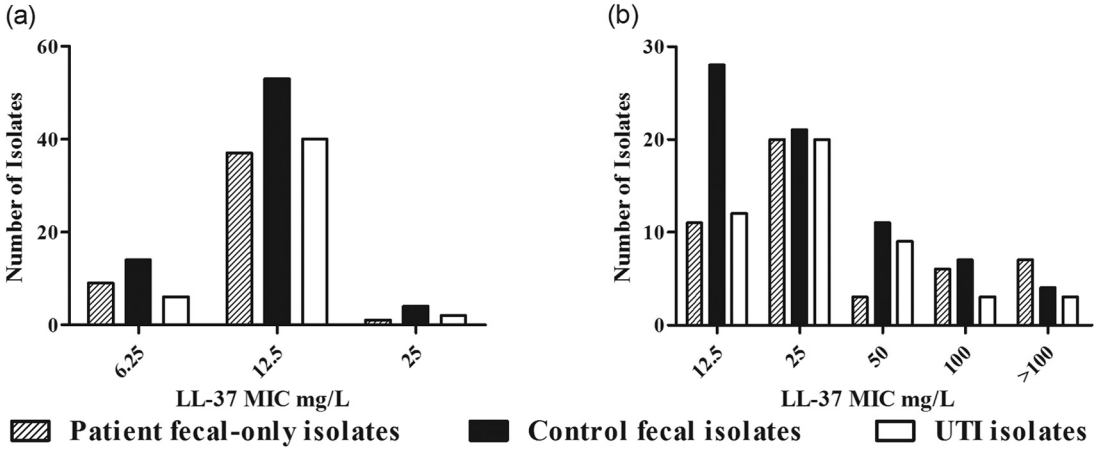

FIG 2 MIC values for LL-37 in broth dilution assays without biofilm promotion (a) and with biofilm promotion (b). In the biofilm-promoting assay, control fecal isolates had a significantly larger proportion of isolates with a MIC of 12.5 mg/liter than the case for UTI and patient fecal-only clones ($P = 0.04$).

obtained during UTI, it was possible to measure the concentration for the majority of the patients (36/47 patients). However, for controls and samples from patients after infection, it was possible to measure the concentration in only 25 of 50 and 15 of 47 samples, respectively. We therefore applied Tobit modeling of the data in order to take missing data into account. Regression analyses showed that the peptide concentration was not correlated with the total number of UTIs experienced by patients (based on medical journals) or with recurrence (data not shown).

There was no significant correlation between the number of CFU/ml in urine and the LL-37 concentration.

LL-37 MIC. Bacterial differences in LL-37 susceptibility may indicate why bacteria are more likely to infect the bladder as part of a complex interplay between host and pathogen. LL-37 MICs for all *E. coli* isolates causing UTI, fecal-only *E. coli* clones (identified by RAPD PCR) from UTI patients, and fecal clones from controls were determined. This assay was performed as both a regular MIC broth dilution assay and a biofilm-promoting broth dilution assay similar to that of Kai-Larsen et al. (24).

MIC values in the assay without biofilm enhancement showed similar levels for UTI isolates, fecal isolates from controls, and fecal-only isolates from patients (fecal clones not causing the current UTI infection) (Fig. 2a). For the biofilm assay, we observed a 2-fold higher MIC distribution of patient fecal-only clones and UTI isolates than that for controls (Fig. 2b). There were significantly more control fecal isolates with a MIC of 12.5 mg/liter than the case for fecal-only clones from patients and UTI isolates ($P = 0.04$) (Fig. 2b).

More phylotype A isolates had a MIC of 12.5 mg/liter than was the case for any other phylotype ($P = 0.01$ by Fisher's exact test) (Fig. 3). LL-37 MIC values of B1 isolates were ≥25 mg/liter; however, these were not significantly different from those for isolates of the other phylotypes ($P = 0.1$) (Fig. 3). LL-37 MIC proportions were similar for phylotype B2, D, and NT isolates.

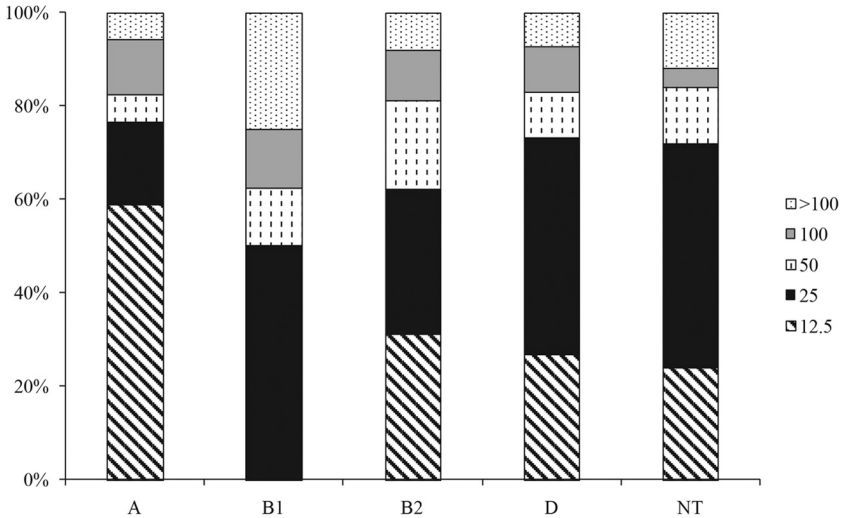

FIG 3 Proportional distributions by phylotype of LL-37 MIC values (mg/liter) in the biofilm-promoting assay. There was a significantly larger proportion of phylotype A isolates with a MIC of 12.5 mg/liter than the case for the other phylotypes ($P = 0.01$ by Fisher's exact test). Phylotype B1 had no isolates with a MIC of 12.5 mg/liter, but it was not significantly different from the other phylotypes ($P = 0.1$).

***In vivo* infection with fecal *E. coli* isolates.** We performed UTI mouse model experiments in order to investigate whether LL-37 susceptibility was linked to *in vivo* virulence of the isolates, as well as to determine whether fecal *E. coli* isolates from both UTI patients and controls could cause infection in the bladder and kidneys. We investigated the virulence of selected isolates, obtained from patients and controls and with high or low LL-37 MIC values, in a UTI mouse model. From the fecal floras of patients and controls, we selected phylotype B2 and D *E. coli* isolates with different LL-37 MICs, i.e., 12.5 mg/liter and ≥100 mg/liter (12 isolates in total). The CFU counts in the urine, bladder, and kidneys were investigated after 3 days of infection (Fig. 4).

The results showed that isolates from both UTI patients and controls were able to cause infection in the urine, bladder, and kidneys (Fig. 4). There was no significant difference in numbers of CFU between the 4 groups of isolates (phylotype B2 and D isolates with high and low LL-37 susceptibilities) for the bladder and kidneys, regardless of LL-37 susceptibility (Fig. 4) ($P > 0.05$ by the Kruskal-Wallis test).

Role of hBD-1 in uncomplicated UTI. Levels of hBD-1 measured in urine were in the range of 3.5 to 316 mg/g of creatinine (Fig. 5). The three groups differed significantly from each other ($P = 0.03$ by ANOVA). Case samples during and after infection proved not to be significantly different ($P = 0.26$ by the two-tailed unpaired *t* test with the Welch and Bonferroni correction). There was a trend, though not a significant one, that baseline levels for UTI patients were higher than those for controls ($P = 0.08$ by the two-tailed unpaired *t* test with the Welch and Bonferroni correction).

Regression analysis correlating the concentration of hBD-1 in each patient to the number of CFU in urine and the creatinine concentration indicated no significant trends (data not shown).

Three SNPs, i.e., G-20A, C-44G, and G-52A, located upstream of the structural hBD-1 gene, were typed by DNA sequencing; the structural gene, including exons 1 and 2, was also sequenced. The structural gene revealed no sequence variations (data not shown). There was no correlation between the three SNPs and the status of being a UTI patient or control. Logistic regression was performed to analyze whether patients with recurring infections had a different distribution of any of the three SNPs compared to patients without recurrence. This analysis indicated no correlation between the SNPs and recurrence of infection. Finally, according to multiple-regression analysis, there was no association between hBD-1 concentration and any of the three SNPs.

DISCUSSION

Human antimicrobial peptides are essential defense molecules against microbial infections, and several studies have suggested that these peptides play an important role in preventing UTI. The current study was performed in an attempt to clarify whether LL-37 and hBD-1 production (or lack thereof) predicts the development of UTI.

This study is, to the best of our knowledge, the first study performed on otherwise healthy, nonhospitalized adult women to compare expression of LL-37 in urine during infection, as well as the first study to compare the baseline levels of LL-37 and hBD-1 in urine for patients postinfection and for controls. The present study found that uncomplicated urinary tract infections cause an increase in urinary LL-37 levels during infection compared to the levels found postinfection. Additionally, we found significantly less LL-37 production in UTI patients postinfection than in a comparable control group that had never had a UTI, indicating that reduced LL-37 production may be associated with developing fewer UTIs. In contrast to the study by Chromek et al. (7), who measured LL-37 levels in urine from children without infection or with cystitis or pyelonephritis, the present study concerns uncomplicated lower UTI in adult women only, based on a narrow definition of patients and controls. Whether the low concentrations of

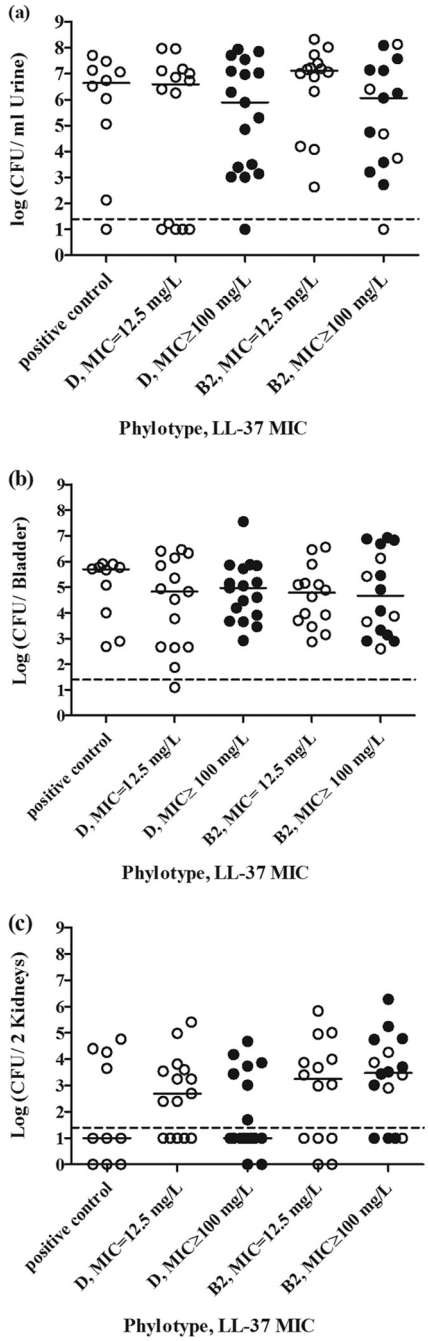

FIG 4 *E. coli* counts in the urine (a), bladder (b), and kidneys (c) in the urinary tract infection mouse model 72 h after inoculation. Each point represents one mouse, and horizontal lines indicate medians. The assay included a positive control, five isolates from patients (closed circles), and seven isolates from controls (open circles). Isolates were of phylotype B2 or D and had a high or low sensitivity to LL-37 (MIC of 12.5 mg/liter or ≥100 mg/liter, respectively).

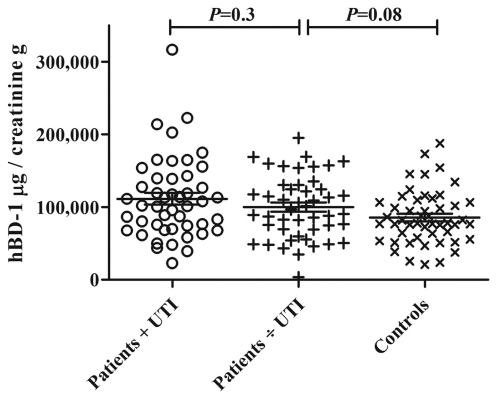

FIG 5 Concentrations of hBD-1 in urine normalized to creatinine concentrations for patients during infection (patients + UTI) and after infection (patients ÷ UTI) and for controls. Horizontal lines and error bars show means ± SEM. ANOVA indicated differences between the three groups ($P = 0.05$), but the posttest indicated no significant difference between values obtained during and after infection, as well as no difference between baseline levels for patients and controls ($P = 0.3$ and $P = 0.08$, respectively, by two-tailed unpaired *t* tests with the Welch and Bonferroni correction).

LL-37 postinfection were due to a rebound effect would need further study, e.g., repeated month-long sampling of urine from UTI patients.

Genital and fecal floras have a strong influence on development of UTI, and the microbiota plays a pivotal role in protection from infections (33). We found a difference in the distribution of LL-37 MIC values in comparing fecal *E. coli* isolates from patients and controls. Furthermore, more patient fecal isolates than control isolates had high MICs when grown in a biofilm. This could indicate better biofilm formation of patient isolates than control isolates, as curli, an important component of biofilm, is known to inhibit the activity of LL-37 (24). Additionally, it could indicate that the *E. coli* fecal flora of patients is more resistant to LL-37 activity than that of controls. We have not investigated the mechanisms behind lower susceptibility further, but it is speculated that this is due to biofilm formation or increased virulence of the isolates.

We have shown that LL-37 MIC distributions vary for the four phylogenetic groups. The finding that phylotype A isolates have less susceptibility to LL-37 than the other phylotypes could indicate that susceptibility to LL-37 is linked to virulence of the isolates, as phylotypes B2 and D are generally considered to be more pathogenic than phylotype A (34). This is in line with our findings of higher MICs in biofilms than during planktonic growth.

We investigated the virulence of fecal isolates from patients and controls in order to determine whether both patient and control isolates could generate UTI and whether a high or low LL-37 MIC had an effect on virulence of the isolate. We found that fecal isolates from both UTI patients and controls were able to cause bladder and kidney infections. These results indicate that while controls carry isolates capable of causing bladder infections, whether those isolates actually cause infection most likely depends on other factors as well, e.g., the interplay between the pathogen and host factors, supporting the other results of this study.

Additionally, we found that the MIC of LL-37 was not predictive of the levels of infection in mouse bladders and kidneys. It has been shown that *E. coli* strains from cases of pyelonephritis have higher MIC values for LL-37 than *E. coli* strains from cases of cystitis (7). Our results show that kidney infection in the mouse model is not dependent on LL-37 MIC values.

The LL-37 peptide was recently investigated by others with respect to urinary tract infection. Transcription of CAMP, encoding LL-37, is regulated by the vitamin D receptor in humans, and it has been shown that vitamin D_3 supplementation can increase LL-37 expression (8, 10). Future studies can reveal whether vitamin D_3 supplementation can be used prophylactically in order to lower the UTI risk in UTI-prone individuals.

hBD-1 has been proven to be increased during pyelonephritis (12), and it is thought to be important for lower urinary tract infections as well. The analysis of urinary hBD-1 indicated that hBD-1 concentrations in urine were similar between lower UTI patients and controls. This peptide is expressed in the distal loop of Henle (6), and hence it is not directly involved in the first line of defense of the lower urinary tract but, rather, that of the upper urinary tract. This could explain the lack of correlation between lower urinary tract infections and hBD-1 production. Sequencing of hBD-1 indicated that there were no sequence differences between patients and controls that could generate alternative cleavage sites. Results of genetic analysis and SNP analysis support the measurements of hBD-1 in urine, as no significant differences were found for patients with cystitis and controls who had never had a urinary tract infection.

We acknowledge that this is a relatively small study and that larger studies are necessary to corroborate the results, although the study participants of this study were very homogenous: all were otherwise healthy persons who were nonhospitalized, of similar age, premenopausal, and sampled from the same study population, which strengthens the conclusions of the study.

In conclusion, we have found a correlation between the LL-37 concentration in urine and uncomplicated urinary tract infections in otherwise healthy premenopausal women with *E. coli* infection. Additionally, we have found that fecal *E. coli* isolates from controls have less susceptibility to LL-37 than patient isolates, further stressing the importance of this peptide for developing UTIs. The results indicate that the concentration of LL-37 in the urinary tract and susceptibility to LL-37 could influence the likelihood of UTI, in a complex interplay between host and pathogen attributes.

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