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ORIGINAL PAPER

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Viable but nonculturable uropathogenic bacteria are present in the mouse urinary tract following urinary tract infection and antibiotic therapy

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Abstract Involvement of the viable but nonculturable (VBNC) condition in recurrent urinary tract infections (UTIs) was investigated. VBNC bacteria are those which are alive but do not give rise to visible growth under nonselective growth conditions. Urine, bladder, and kidney samples collected over a 2-month period from BALB/c mice inoculated with the uropathogenic *Escherichia coli* strain J96 were examined to determine the level of culturable and viable bacteria. Urine from uninoculated mice was found to contain more viable than culturable bacteria. Inoculated mice had a transient increase in the level of culturable forms of the uropathogen in their urine, followed by a decrease to background levels; they also had multiple log higher levels of viable cells than culturable cells. The culturable pathogenic bacteria in mice that were inoculated and received antibiotic treatment dropped to undetectable levels within 1 week. At 2 out of 12 subsequent time points spanning an additional 65 days, culturable forms of the inoculated pathogenic bacteria were recovered. Polymerase chain reaction (PCR) analysis confirmed that DNA from the inoculated bacteria was present in a sample that yielded no culturable bacteria. These data indicate that the inoculated uropathogenic *E. coli* was not eliminated by antibiotic therapy, and suggest that these bacteria may escape detection by current standard culturability assays because they are VBNC.

Key words Urinary tract infection · Mouse · Antibiotic treatment · Viable but nonculturable · *Escherichia coli*

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Introduction

Urinary tract infections (UTIs) affect over 6 million people, mostly women, annually [11, 13]. Both gram-negative and gram-positive bacteria cause UTIs; however, *Escherichia coli* accounts for between 85 and 90% of all cases [1, 2, 10]. UTIs are diagnosed both on the basis of patient symptoms and by quantitative cultures of urine. Traditionally, a threshold of 100,000 colony-forming units (CFUs)/ml of clean-catch urine is used to diagnose a UTI [1]. However, this concentration of culturable bacteria is not a definitive indicator of a UTI. Some patients present with a "low count" bacteriuria, which has been suggested to be a sign of a developing or subclinical UTI [11], and others present with urinary tract symptoms with no culturable bacteria in the urine [13].

An uncomplicated UTI is usually treated successfully with antibiotics. Although the bacteria appear to be eradicated from the urinary system of the patient, most women diagnosed with a UTI will have a subsequent infection [1, 16]. Because the bacteria causing subsequent infections are usually not identical to the primary strain [1], most recurrent infections are likely to be caused by reinfection of the urinary tract. For the remaining cases, bacterial invasion of urinary epithelial cells [14] or the formation of biofilms [4] could serve as foci for subsequent persistent infections. We propose that recurrent infections caused by the same index strain could escape detection during asymptomatic periods by the bacteria entering the viable but nonculturable (VBNC) condition in vivo.

The VBNC state is defined as one in which cells are viable and yet do not undergo sufficient division to give rise to visible growth on nonselective growth medium [3]. This condition has been reported to occur in many gram-negative bacteria including human pathogenic *E. coli* [12]. Cells are induced to enter the VBNC state by one or a combination of environmental stresses, including starvation, temperature shift, and exposure to a heavy metal [5]. To document viability, the most com-

mon microscopic-based assays measure metabolic activity (such as through the observation of chemical reduction of redox dyes [15] or the observation of cell elongation in the presence of nutrients and a topoisomerase inhibitor [9]) or detect the presence of an intact cell membrane [7]. What distinguishes the dormant-like VBNC state from similar bacterial cell responses such as cell stress, cell starvation, and cell wounding is that VBNC cells can remain in this condition for long periods of time. In addition, only VBNC cells do not give rise to visible growth when placed under nonselective growth conditions. The ability of VBNC cells to regain the ability to grow, so-called resuscitation, has only recently been documented [12].

The purpose of this study was to determine whether evidence could be obtained of the presence of VBNC cells in the urinary tract of mice following infection with a UTI-causing strain of *E. coli* and subsequent antibiotic treatment.

Materials and methods

Bacterial strains and chemicals

ES98, a spontaneous rifampicin-resistant mutant of *E. coli* strain J96 [6] transformed with pGFPmut3.1 J96 (O4, K6), is a motile, hemolytic human pyelonephritis isolate. pGFPmut3.1 is an ampicillin-resistant plasmid containing a mutant *gfp* gene encoding a long half-life green fluorescent protein (GFP) under the control of a constitutive lac promoter (Clontech; Palo Alto, Calif., USA). All chemicals were purchased from Sigma (St. Louis, Mo., USA) unless otherwise noted.

Mouse inoculation

Female BALB/c mice aged 4–7 weeks (Charles River Laboratories, Research Triangle Park, N.C., USA) were used in all studies. A maximum of ten mice was housed by experimental group in cages. They were allowed food pellets and water ad libitum and were exposed to 12-h alternating light and dark cycles.

For inoculation into mice, ES98 was passaged twice on Luria-Bertani (LB) agar containing rifampicin (50 μg/ml) and ampicillin (50 μg/ml), then grown overnight at 37 °C in Luria Broth containing both antibiotics. The cells were collected by centrifugation at 13,000 ×*g* for 5 min, and suspended in sterile 0.9% NaCl to a final concentration of 1×10^{11} cells/ml.

General anesthesia was induced with 2.5% Avertin at a dose of 0.015 ml/g body weight IP. Bacteria were administered via catheterization using PE-10 tubing stretched to an approximate outer diameter of 0.3 mm. Each anesthetized mouse was placed in the supine position, and the abdominal and perineal areas were cleaned with 70% ethanol. The tubing was advanced into the urethra approximately 0.5 cm while applying gentle countertraction to the urethral orifice with sterile forceps. A total of 10 μl of the bacterial suspension was slowly injected into the urethra (approximately 1–2 μl/s). After removal of the catheter, the mouse was kept supine for 5 min and then returned prone to a clean cage; all mice were periodically observed until they had fully recovered from the anesthesia.

Buprenorphine (Nubain) was administered b.i.d. by IP injection at a dose of 1 mg/kg body weight [0.3 mg/ml in sterile phosphate-buffered saline (PBS)] for 7 days following the procedure to control discomfort caused by the catheterization and UTI.

Trimethoprim (TMP) was combined 1:5 (w:w) with sulfamethoxazole (SMZ) to produce a TMP-SMZ combination. This was then mixed with dextrose such that 10 mg of the final mixture was equivalent to 0.5 mg TMP-SMZ. Each 30-mg TMP-SMZ/kg body weight dose was mixed with a small portion of CheeseWhiz Processed Cheese food. The CheeseWhiz-drug combination was placed into the mouth of each mouse b.i.d. using a spatula on days 3, 4, and 5. The mice were observed to have eaten each dose of antibiotics.

The Institutional Animal Care and Use Committee at the University of North Carolina at Charlotte approved all experiments involving animal use in this study as described in Protocol 98/99–03.

Urine collection

Urine samples were collected from living mice for 3 days prior to catheterization, immediately preceding anesthesia, and each day thereafter until 5 days after antibiotic treatment was completed. Urine was then collected, from the available mice, at least weekly for a total of 2 months. To obtain urine samples from living mice, each mouse was held over a UV-sterilized plastic weigh dish. Gentle pressure was applied to the abdomen and the urine was collected in the weigh dish without the mouse coming into contact with the container. The first few microliters of urine were discarded.

Kidney and bladder isolation

Five mice from each experimental group and two from the control group were killed by CO_2 overdose on days 3, 7, 13, and 71 to enable the assaying of the bacterial load of the bladder and kidney. Both kidneys and the bladder were aseptically resected and sagitally sectioned. Half of both the right and the left kidneys were pooled. A weighed portion of each organ was placed into 1.5-ml microcentrifuge tube containing 250 μl of sterile Tris-buffered saline (TBS; 137 mM NaCl, 2.7 mM KCl, 25 mM Tris, pH 7.4) and homogenized manually with a Kontes Pellet Pestle (Fisher Scientific, Pittsburgh, Pa., USA). The homogenized organs were kept at 4 °C until microscopy studies could be performed, then placed at −20 °C until used for polymerase chain reaction (PCR) analysis.

Culturability assay

For urine analysis, the volume of each urine sample was recorded and then increased to 500 μl with 0.9% NaCl. A total of 40 μl of each sample was spotted in triplicate onto Luria-Bertani (LB) agar. Plates were incubated at 37 °C for 48 h prior to scoring. Single colonies of culturable bacteria were placed onto LB agar containing 50 μg/ml rifampicin and incubated at 37 °C for up to 48 h prior to scoring.

For tissue analysis (see below), 0.25 ml of TBS was added to each tissue homogenate and vortexed. The samples were then centrifuged at 200 ×*g* for 1 min to pellet nonbacterial debris. Samples were evaluated for total culturable cells and rifampicin-resistant cells as described above. Enumeration of bacteria was calculated as CFUs/g of wet tissue.

Microscopy and viability assay

For urine analysis, each urine sample was examined microscopically for cells containing the stable GFP and for the total number of viable cells. To examine for cells containing GFP, 100 μl of each urine sample was collected onto an 0.22-μm black polycarbonate filter (Osmonics, Livermore, Calif., USA), washed with 1 ml of 0.9% NaCl, and examined with an Olympus BX60 epifluorescent microscope utilizing a HBO103 W/2 Mercury burner. The microscope is equipped with a Spot Cooled Color Digital Camera (Diagnostic Instruments) controlled by SpotCam (Diagnostic Instruments) software used to obtain photomicrographs.

To examine for the total number of viable cells, the *Bac*Light LIVE/DEAD Bacterial Viability Kit (Molecular Probes, Eugene, Ore., USA) was used as per manufacturer's instructions. Briefly, 0.25 ml of each sample was incubated with the kit reagents (using

1 µl of reagent A and 2 µl of reagent B) in the dark for approximately 1 h. It was then collected onto 0.22-µm black polycarbonate filters, washed with 5 ml of 0.9% NaCl, and examined microscopically. The number of red and green rod-shaped cells per field was recorded. The concentration of cells that would give rise to even 1 cell/10 fields of vision was dependent upon the initial sample volume but generally was at least 1e4 cell/ml.

Each homogenized tissue sample was examined microscopically for the total number of viable cells as described above with the addition that stained cells were passed through a 2.4–6 µm borosilicate microfiber glass pre-filter (Millipore, Bedford, Mass., USA) to remove debris prior to collection onto 0.22-µm black polycarbonate filters.

PCR analysis of *gfp*

Primers were chosen such that a region of the GFP gene (*gfp*) could be amplified. The sequence of plasmid pGFPmut3.1 was obtained from Clontech (Palo Alta, Calif., USA), and the open reading frame of *gfp* was entered into an online program called Primer3. A pair of primers complementary to a region of *gfp* was selected using the online program. The primers were synthesized by Genemed Synthesis (San Francisco, Calif., USA). The sequence of the forward primer is 5′-CAGTGGAGAGGGTGAAGGTG-3′, and the sequence of the reverse primer is 5′-AAAGGGCAGATTGTGTGGAC-3′. The gene product amplified using these primers has 536 base pairs.

The homogenized bladder and kidney tissues from each mouse were lysed by the addition of 1/9 volume of a proteinase K solution to each sample [proteinase K solution: 4 ml of proteinase K (10 mg/ml in TE 10 mM Tris [pH 8.0], 1 mM EDTA) was added per 0.7 ml digestion buffer containing 50 mM KCl, 10 mM Tris (pH 8.3), 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% tergitol NP-40, and 0.45% Tween-20 immediately prior to use]. The samples were incubated for 1 h at 55 °C and 10 min at 95 °C.

All PCR reagents were obtained from Promega (Madison, Wis., USA). Reactions with 25 µl of sample were carried out in 0.6-ml microcentrifuge tubes containing one 1.25 U TaqBead. A total of 110 ng of the forward primer and 5 µl of PCR master mix [1.25 U Taq DNA polymerase, 10 mM dNTPs, and 5 × PCR buffer without magnesium] were added to each tube. The tubes were incubated at 85 °C for 2 min, then allowed to cool to room temperature. A total of 12.5 µl of lysate, 1.5 µl of sterile deionized water, 110 ng of the reverse primer, and 4 µl (4 mM) magnesium chloride were added on top of the wax. The tubes were incubated at 85 °C for 1 min, and then subjected to 40 cycles of 90 °C for 1 min, 56 °C for 2 min, and 72 °C for 2 min using a Techne PHC-1 thermocycler. The final extension step at 72 °C was continued for an additional 10 min. Samples were then analyzed by agarose gel electrophoresis in a 2% agarose gel in 1 × TAE (40 mM Tris base, 20 mM Na acetate, 1 mM EDTA-Na_2, pH 7.2).

Results

VBNC bacteria are present in the urine of uninfected mice

Before examining urine from mice infected with a UTI-causing strain of *E. coli*, the level of culturable and viable bacteria present in uninfected mouse urine was determined. Clean-catch urine was collected from ten BALB/c mice, as described in the Materials and methods section. The level of culturable bacteria was determined by spotting a sample of urine onto LB growth agar plates, then counting the number of visible colonies present after incubation at 37 °C for at least 2 days. The average level of culturable bacteria present in urine is shown in Fig. 1. Consistent with what was expected, few culturable bacteria were detected. The range in the average concentration of culturable bacteria/ml of urine ranged from no detectable bacteria (for two of the seven time points) to 5×10^3 CFU/ml. Because the pathogenic bacteria we used for subsequent inoculation experiments carries a gene encoding resistance to the antibiotic rifampicin, the concentration of rifampicin-resistant bacteria present in urine of uninfected mice was determined. Colonies from LB plates lacking rifampicin were transferred to LB plates containing the antibiotic. None of the bacteria grew on media containing rifampicin (Fig. 1).

The level of viable bacteria was determined using the *Bac*Light LIVE/DEAD Bacterial Viability Kit as described in the Materials and methods section. This assay uses two fluorescent dyes with different membrane permeabilities to distinguish those cells with an intact membrane (i.e. viable) from those lacking such (i.e., dead). The average level of viable bacteria present in urine is shown in Fig. 1. Surprisingly, the level of viable bacteria was multiple logs greater than the level of LB-culturable bacteria. The average level of viable cells ranged from less than 5×10^5 to 8×10^7 cells/ml.

Urine from mice infected with pyelonephritic *E. coli* contains viable bacteria not culturable on LB

To examine the relationship between VBNC bacteria and murine UTIs, the pathogenic bacteria that were to be inoculated into mice were genetically modified to allow them to be distinguished from indigenous bacteria. Spontaneous rifampicin mutants of strain J96 were isolated. This strain was then transformed with pGFPmut3.1. This plasmid harbors a gene encoding a

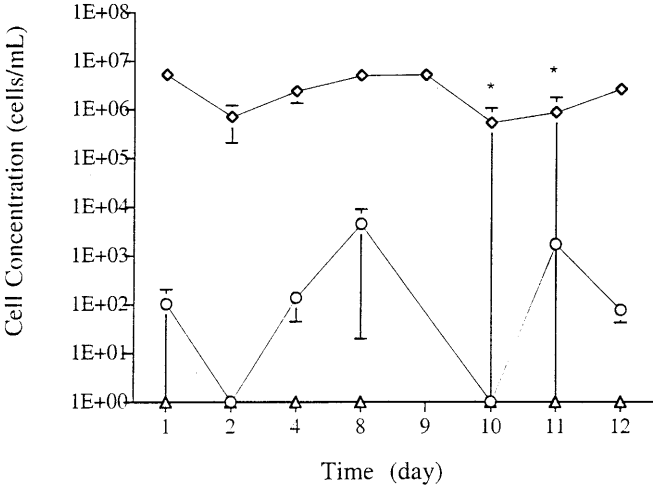

Fig. 1 Concentration of culturable and viable cells in urine from uninfected mice. Urine collected from ten mice was measured for levels of culturable cells (○), rifampicin-resistant culturable cells (△), and viable (◇) cells as described in the Materials and methods section. *Error bars* indicate the SEM. * For this sample, no cells were observed in the viability assay. The value used for graphing represents the limit of detection for determining the viable cell concentration (see Materials and methods section)

long half-life form of the GFP. Cells of this strain, designated ES98, were then instilled into the urethra through a catheter as described in the Materials and methods section. During experiments to determine the number of bacteria required to induce UTI infection, it was repeatedly observed that rifampicin-resistant bacteria recovered from the urine of infected mice lost the ability to fluoresce within 1 day of inoculation. Hence, the GFP protein could not be used to microscopically differentiate between indigenous and inoculated bacteria.

For the subsequent experiments, mice were subjected to one of three conditions: inoculation with sterile saline, inoculation with ES98, or inoculation with ES98 followed by antibiotic treatment. The experimental design, and the number of mice used, was as follows. Using ES98, UTI was achieved in 40 mice by inoculation with 1×10^9 cells (in a total volume of 0.01 ml) as described in the Materials and methods section. Twenty of these mice received six doses of the antibiotic combination TMP-SMZ over 3 days, beginning after the infection had fully developed (i.e. beginning on day 3). The remaining 20 mice received no antibiotics. All mice received twice daily injections of buprenorphine. Eight control mice were inoculated with sterile saline. The number of culturable and viable cells in the urine was monitored over a 72-day time span. For the control mice, the pattern of culturability (Fig. 2) was similar to the background levels shown in Fig. 1; the concentration of culturable cells ranged from 10,000 CFU/ml of urine to nondetectable levels.

For the mice inoculated with bacteria, the number of culturable bacteria from both the antibiotic treated and untreated groups was approximately 1×10^6 CFU/ml of urine 24 h after inoculation (Fig. 2). For the inoculated mice not receiving antibiotic treatment, the level of culturable bacteria remained above control levels for approximately the first 14 days, then dropped to a level within the high range of the control group. For the mice receiving antibiotic therapy, the concentration of culturable bacteria decreased to less than 1×10^3 CFU/ml of urine within 2 days of completing the treatment, and then remained within the high range of the control group's level.

To determine whether the culturable bacteria recovered from urine represented the inoculated cells, colonies that grew on the nonselective LB agar were placed on LB agar containing rifampicin. During the first 5 days the concentration of rifampicin-resistant bacteria for the infected mice was similar to that of total bacteria (Fig. 3). However, beginning on day 8, a large difference in the concentration of rifampicin-resistant bacteria was observed between the two infected groups. For the mice that did not receive antibiotics, the inoculated bacteria were present at high levels throughout the experiment. For the mice receiving TMP-SMZ, no rifampicin-resistant bacteria were detected on day 8 or for the remainder of the experiment, with two exceptions (discussed below). No rifampicin-resistant bacteria were recovered from uninoculated mice.

The concentration of viable cells revealed that there was no significant difference between the three groups of mice (Fig. 4). The concentration of viable bacteria was usually within the range of 1×10^6 to 1×10^7 cells/ml of urine.

Examination of bacteria in the bladder and kidney

The fact that rifampicin-resistant bacteria could be recovered from the urine for over 2 months following inoculation suggested that the bacteria had colonized the urinary tract. To determine whether cells were colonizing the bladder or kidneys, these organs were removed from five mice from each of the inoculated groups (with and without antibiotic therapy) and two mice from the sham group on days 3, 7, 14, and 71 following inoculation. The organs were homogenized, bacteria were

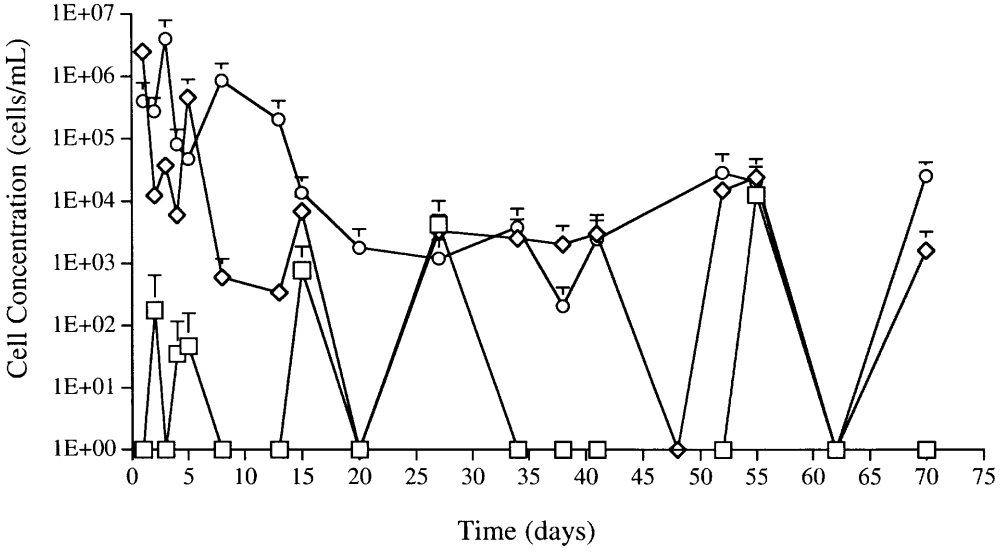

Fig. 2 Concentration of culturable cells in urine from inoculated mice. Levels of culturable cells were measured in urine collected from uninoculated mice (□), inoculated mice receiving no antibiotics (○), and inoculated mice receiving TMP-SMZ (◇) as described in the Materials and methods section. *Error bars* indicate the positive SEM

isolated as described in the Materials and methods section, and both culturability and viability were determined as previously described. When the bladder was examined, no culturable or viable cells were observed in the tissue of the control mice. Culturable bacteria at a concentration of between 1×10^3 and 1×10^5 rifampicin-resistant cells/g of tissue were recovered from both infected mice groups on days 3, 7, and 14, but none were recovered on day 71. Viable cells were observed in both inoculated mouse group samples on days 3 and 7, at a concentration of between 1×10^4 and 2×10^5 viable cells/g of tissue, but none were observed on days 14 and 71 (data not shown). The fact that some tissue samples contained detectable culturable cells but no detectable viable cells is likely to be the result of the viability assay having a level of detection of at least 1×10^3 cells/g of tissue (see Materials and methods section).

Similar results were obtained with kidney samples; however, fewer cells were observed. Between 50 and 2×10^3 rifampicin-resistant cells/g of kidney were recovered from infected mice only on days 3, 7, and 14. Viable cells were detected in the kidney only from day 3 in inoculated mice that received antibiotics (data not shown).

Evidence for resuscitation of inoculated *E. coli*

Results from the inoculated mice receiving antibiotics, in which culturable cells were observed at only 2 of the last 12 urine time points while high levels of viable cells were present at every time point, are consistent with the inoculated cells being present in the urinary tract in the VBNC state (Figs. 3, 4). The results from the bladder and kidney analysis suggested that either the inoculated cells had not colonized the bladder or kidney or that the cells had colonized these organs and were present in the VBNC state (albeit at a level that was too low to be detected by the viability assay).

To determine whether the inoculated cells were present in these tissues, PCR was conducted as described in the Materials and methods section using the *gfp* gene as the target. This gene was chosen because it is the only

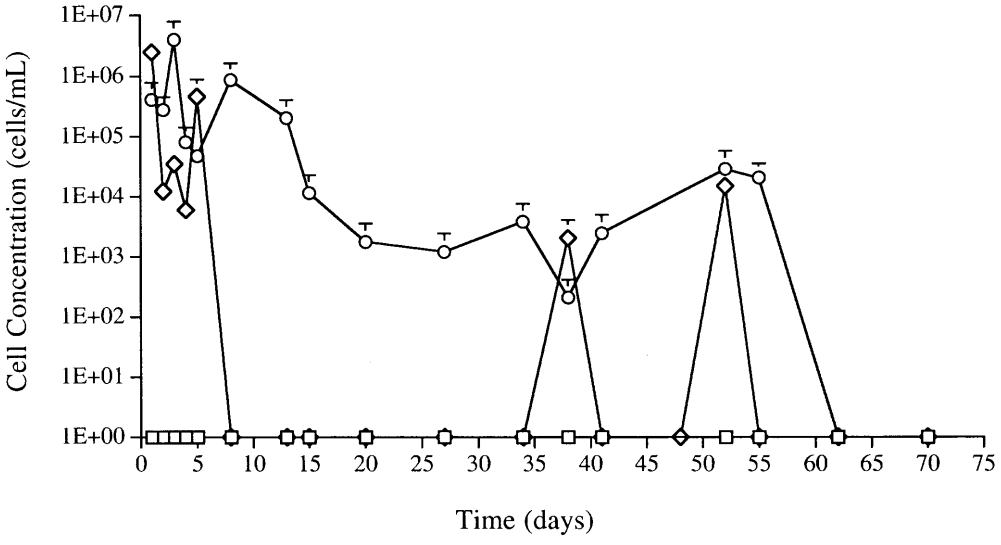

Fig. 3 Concentration of rifampicin-resistant cells in urine from inoculated mice. Levels of rifampicin-resistant culturable cells were measured in urine collected from uninoculated mice (□), inoculated mice receiving no antibiotics (○), and inoculated mice receiving TMP-SMZ (◇) as described in the Materials and methods section. *Error bars* indicate the positive SEM

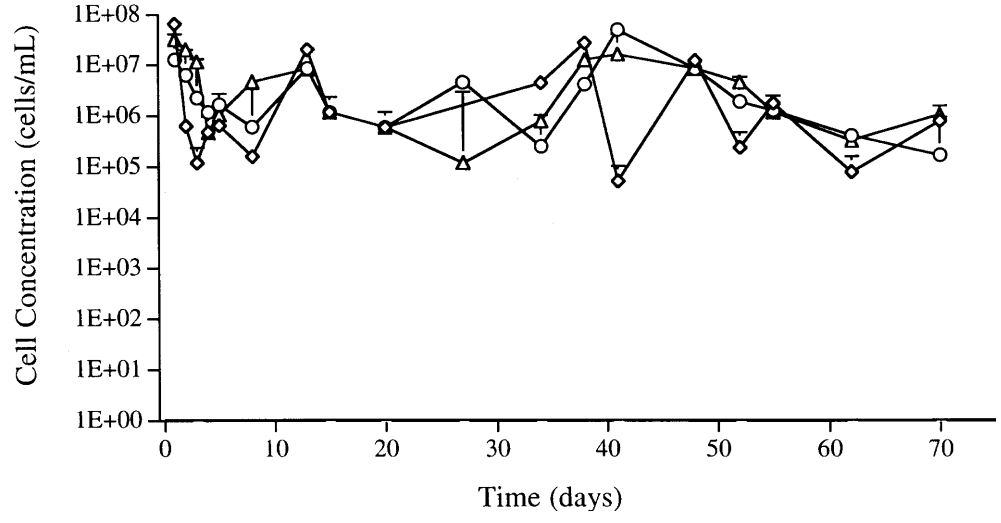

Fig. 4 Concentration of viable cells in urine from inoculated mice. Levels of viable cells were measured in urine collected from uninoculated mice (○), inoculated mice receiving no antibiotics (□), and inoculated mice receiving TMP-SMZ (◇) as described in the Materials and methods section. *Error bars* indicate the positive SEM.
* For this sample, no cells were observed in the viability assay. The value used for graphing represents the limit of detection for determining the viable cell concentration (see Materials and methods section)

DNA sequence known to be absent in the indigenous bacterial population. Initial experiments were conducted to determine the minimum number of cells necessary to observe an amplification signal. Controls to determine sensitivity were conducted using urine samples to which various numbers of culturable rifampicin-resistant bacteria were added. A signal could be seen with as few as 100 cells, but at least 10,000 cells were required to obtain a signal approximately 85% of the time.

Amplification reactions were performed on homogenized kidney and bladder tissue removed from inoculated mice with and without antibiotic therapy at days 3, 7, 14, and 71 after inoculation. Five mice were examined per condition per time point. For tissues that yielded an amplification product, a second sample was also analyzed. Results from an amplification experiment showing a positive and a negative result are given in Fig. 5. A reproducible PCR signal was seen from the bladder of an inoculated mouse not receiving antibiotics (day 3) and from the bladder of a mouse receiving antibiotics (day 71).

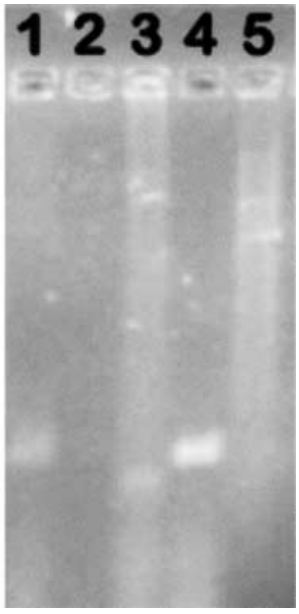

Fig. 5 PCR amplification of *gfp* isolated from the bladders and kidneys of infected mice. These organs were removed at various time points from mice inoculated with J96 and not receiving antibiotics as described in the Materials and methods section. DNA isolated from homogenized tissue was subjected to PCR amplification of a region of the *gfp* gene located on a plasmid as described in the Materials and methods section. The amplification products were then subjected to electrophoresis as described in the same section. *Lane 1*, day 3 bladder; *lane 2*, day 71 kidney; *lane 3*, day 71 bladder; *lane 4*, positive control DNA; and *lane 5*, day 14 bladder. The *arrow* marks the position of the expected amplification signals that are seen in *lanes 1* and *4*

Discussion

Most women diagnosed with a UTI will become reinfected [1, 16]. Some recurrent UTIs are caused by the same index strain [1], suggesting that the original pathogenic strain may not have been completely eliminated by antibiotic therapy. Consistent with the observation that reinfection can be caused by the initial strain, even after the patient has completed successful antibiotic therapy and no culturable cells are detected, is the fact that recurrent UTIs may also arise from pathogenic cells existing in the urinary tract in a nonculturable form. To test this hypothesis, we have investigated whether the presence of VBNC cells in mice is correlated with the presence or absence of UTIs.

Initial experiments that characterized the urine of uninfected mice found evidence for the presence of VBNC bacteria. The concentration of viable cells was measured using the *Bac*Light Bacterial Viability Kit, which differentiates between cells having an intact membrane (i.e., viable) and those having a compromised membrane (i.e., dead). The concentration of viable cells was found to be multiple logs higher than that of bacteria that could be cultured on LB (Fig. 1). The difference in concentration of viable and culturable cells is often used to calculate the concentration of VBNC cells. In this case, there are two reasons why the concentration of VBNC cells may actually be less than the difference in concentration between viable and culturable cells. First, we quantitated only the concentration of cells that would give rise to visible colonies on LB incubated under aerobic conditions. It is possible that additional culturable bacteria were present that could not grow under these conditions. However, it is doubtful that this accounts for much of the observed difference in viability and culturability. In subsequent experiments we have not observed a significant difference in the concentrations of bacteria in urine cultured on LB and brain heart infusion (BHI) media incubated under aerobic and anaerobic conditions (unpublished observations). Second, it is possible that the viability assay may have counted as viable dead bacteria whose cell membrane had not broken down sufficiently to allow an indicator dye to enter the cell. The time it takes for a bacterial cell membrane to break down is dependent upon multiple variables; however, it is likely that conditions found in the urinary tract of a mouse (aqueous environment, high temperature) would favor fast membrane breakdown.

To determine whether the VBNC condition could be involved in the etiology of persistent UTIs, the urine, kidneys, and bladders of mice inoculated with a rifampicin-resistant uropathogenic strain of *E. coli*, and either treated with antibiotics or not, were examined for the levels of viable and culturable cells. Regardless of the infectious status of the mice, the level of viable bacteria in urine was multiple logs higher than the level of culturable bacteria. Examination of the latter in the urine of infected mice indicated that without antibiotic therapy, the levels of inoculated pathogenic bacteria dropped to background levels (Fig. 2). These results confirm a previous report that BALB/c mice are able to clear a UTI infection within approximately 2 weeks [8]. They also extend those findings by showing that the mice do

so not by completely eliminating the uropathogenic bacteria from the urinary tract but by reducing the number of pathogenic bacteria to levels found in uninfected mice. For inoculated mice receiving antibiotic therapy, the number of culturable rifampicin-resistant bacteria was reduced to undetectable levels within 1 week of antibiotic treatment. The level of viable bacteria remained unaffected by antibiotic treatment; levels were above background only during the first week after inoculation, then decreasing to background levels.

The data presented in this manuscript are the average of 8–20 mice per data point for Figs. 2 and 3, and 2–5 mice per data point for Fig. 4. The high standard error of the mean (SEM) values given in these figures indicate that the absolute number of viable and culturable cells present in the urine was not the same in all of the mice. This observation is not surprising and indicates that there can be variation in the absolute bacteria-carrying load in different mice during and after an infection.

The lack of culturable, rifampicin-resistant bacteria after antibiotic therapy would indicate that the inoculated uropathogenic bacteria were eliminated from the urinary tract. However, two experimental results suggest that these bacteria were present within the urinary tract in the VBNC state. First, rifampicin-resistant bacteria were recovered from urine at two later time points (days 38 and 53; Fig. 3). Second, PCR analysis indicated that the *gfp* gene, found only in the inoculated cells, was present in at least one kidney and bladder tissue sample in which no culturable bacteria were detected (Fig. 5). The fact that this signal was not detected in all samples taken at subsequent time points is likely to be the result of the lack of adequate sensitivity of our PCR amplification.

We cannot estimate the concentrations of VBNC pathogenic bacteria present in the urinary tract of mice because we cannot identify different bacterial strain types when performing the viability assay. However, these data are consistent with the existence of VBNC bacteria in the urine of mice, and the fact that pathogenic bacteria can become VBNC following antibiotic treatment. Because it is doubtful that bacteria would remain only in the urine of a mouse for over 2 months, the presence of pathogenic bacteria in the urine of mice almost 2 months after inoculation suggests that the inoculated bacteria successfully colonized the urinary tract.

Involvement of the VBNC condition in recurrent infection has not previously been documented. The data presented here indicate that uropathogenic bacteria can escape detection by standard culturability assays, and that they may do so by becoming VBNC. In addition, nonculturable bacteria are able to establish a stable population within the urinary tract. It is possible that these populations of nonculturable bacteria could serve as the source for culturable bacteria causing subsequent infections. This possibility is currently under investigation.

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