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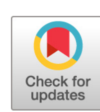

Bacterial Growth of Uropathogenic *Escherichia coli* in Pooled Urine Is Much Higher than Predicted from the Average Growth in Individual Urine Samples

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ABSTRACT Urinary tract infections (UTIs), mostly caused by uropathogenic *E. coli* (UPEC), affect most women, and often recur. Genomic and transcriptomic analyses have not identified a common set of virulence genes, which has suggested complex host-pathogen interactions and multiple virulence mechanisms. One aspect of the host-pathogen interaction is rapid UPEC growth in urine *in vivo*. When bacterial growth in urine is studied *in vitro*, urine is pooled, which is assumed to diminish individual variation. We grew one nonpathogenic and two pathogenic *E. coli* strains in urine from individuals who never had a UTI, had a UTI history but no current infection, and had a UTI history with a current infection. Bacterial growth showed large variations in individual urine samples, and pooled urine often supported significantly more growth than the average growth from individual urine samples. Total nutrient content tended to be higher in current group urine samples than the never and history grouped samples urine. We propose that pooling optimizes a nutrient mixture in the never and history group urine samples, which are often studied, whereas urine from current group individuals may have a more optimal nutrient mixture because of additional nutrient sources. We conclude that a pooled urine is not "an average urine sample," and that the best comparisons of results between labs using pooled urine would also include results with a standardized synthetic urine.

IMPORTANCE Urinary tract infections (UTIs) will affect most women, can recur especially in postmenopausal women, and can become antibiotic recalcitrant. *Escherichia coli* causes most community-acquired UTIs and recurrent UTIs. Current theories of virulence, based on studies of UTI-associated *E. coli*, propose multiple virulence mechanisms and complex host-pathogen interactions. Studies of bacterial growth in urine samples—one aspect of the host-pathogen interaction—invariably involve pooled urine that are assumed to eliminate variations between individuals. Our results show that a pooled urine is not necessarily an average urine sample, and we suggest that quantitative and qualitative variations in nutrient content are the basis for this discrepancy. Knowledge of growth-promoting urinary components is important for understanding host-pathogen interactions during UTIs and could contribute to developing nonantibiotic-based therapies.

KEYWORDS *Escherichia coli*, bacterial growth, urinary tract infection

UTI progression involves several processes, including rapid bacterial growth in urine (1). The failure of recent genomic and transcriptomic analyses to identify a common set of virulence factor genes in UPEC led Hultgren and colleagues to propose that UPEC strains have multiple virulence mechanisms that are based on a complex interplay between bacterial and host properties (2). Urinary composition is one component of the host-pathogen interaction which *prima facie* is a vital component. Recent studies that involve growth in urine have used pooled urine from 2 to 10 healthy individuals (average 5.6) (3–10). Pooling is assumed to eliminate variations between individuals which implies limited variation. This assumption

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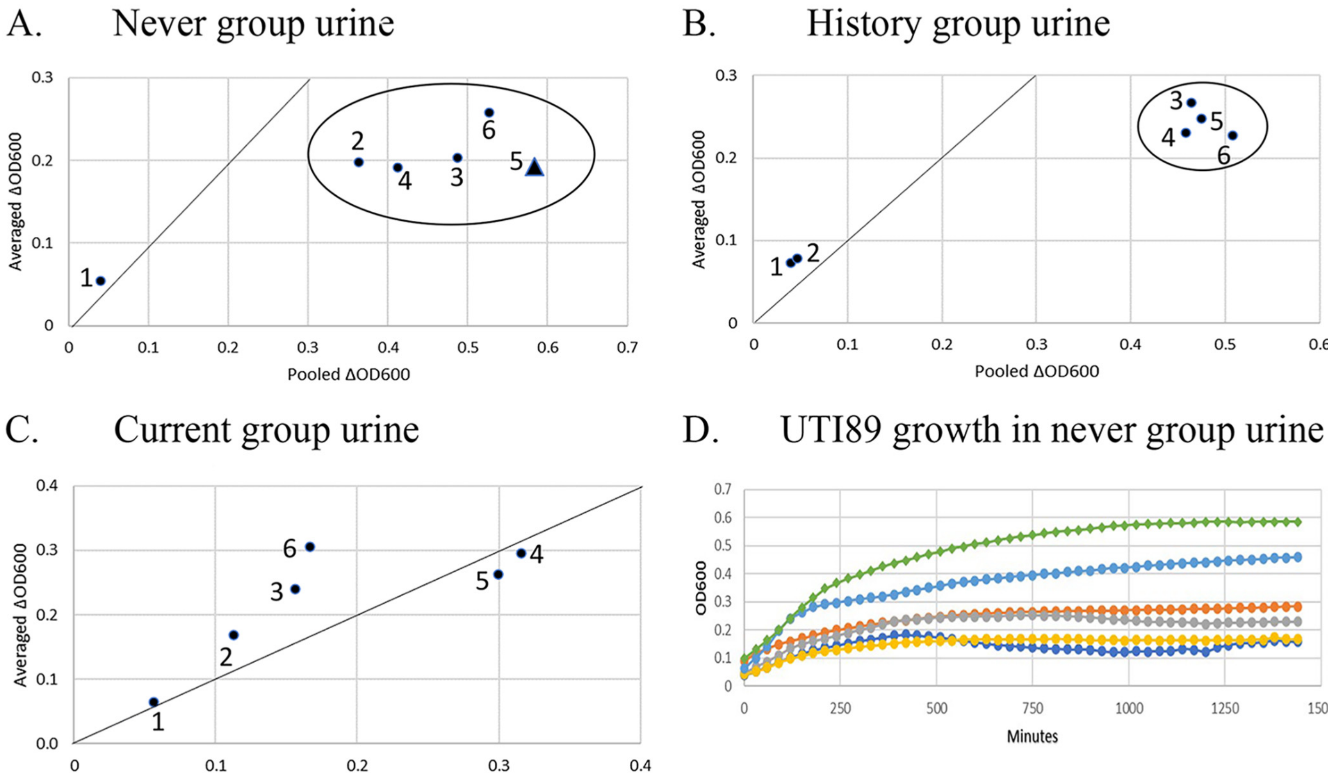

FIG 1 Comparative growth in pooled urine versus the average of individuals samples. (A) Growth in the never group urine; (B) growth in the history group urine; and (C) growth in the current group urine. (D) Growth of UTI89 in the never group urine with a high inoculation density. The numbered labels refer to the strains tested and initial inoculation density: 1, W3110 low; 2, W3110 high; 3, UTI89 low; 4 UTI89 high; 5, LRPF007 low; and 6, LRPF007 high. For the circled symbols, growth in pooled urine was significantly different ($P < 0.05$) from the average growth of the individual samples. The triangle in section A is from the growth curves shown in section D. Each group had five samples, and all growth cultures were performed in triplicate. The average growth in the individual urine samples was from the average of the 5 triplicate averages. For statistical comparisons, the three separate cultures for the pooled urine were compared to the 5 triplicate averages for the individual urine cultures. F-tests were used to analyze if variance was unequal between the individual versus pooled urine. If the F-test was significant, the heteroscedastic *t* test was used to test for differences in the means; if the F-test was not significant, the homoscedastic *t* test was used. The diagonal line is expected if the ratio is one, i.e., growth in pooled urine is the same as the average of the individual urine samples.

predicts that growth in a pooled urine will be similar to the average growth in individual urine samples. To evaluate this prediction, we compared growth in pooled urine to the average growth from individual samples. Urine samples were collected from five postmenopausal women in "never" (never had a UTI), "history" (have a history of UTIs but asymptomatic and negative urinalysis), and "current" (have a history of UTIs and an active symptomatic UTI) groups. We grew three bacterial strains in each urine sample: W3110, a nonpathogenic lab strain; UTI89, a model UPEC strain (11); and LRPF007, a recently isolated UPEC strain from a patient with recurrent UTIs. We analyzed the ΔOD600 as a measure of growth instead of doubling time because few cultures had a constant exponential growth rate for more than one generation even with 30-minute sampling intervals. The ratio of growth in pooled urine versus the average growth in individual urine samples often deviated from 1.0 (the diagonal lines in Fig. 1A to C). The greatest deviations were from growth of the two UPEC strains in never and history group urine samples, and the differences were statistically significant ($P < 0.05$). UPEC growth in pooled urine could be greater than growth in any single urine sample (Fig. 1D).

To understand the basis for the growth synergism, we examined the two general types of factors that affect bacterial growth in urine: inhibitory factors and nutrient content. A characteristic feature of growth inhibitory factors (IFs), such as antimicrobial peptides and antibiotics, is the so-called inoculation density effect, i.e., the initial inoculation density determines the final cell density (12). A pronounced inoculation effect is apparent for W3110 and UTI89 with the antimicrobial peptide LL-37 at 2 and 4 μM (Fig. 2A

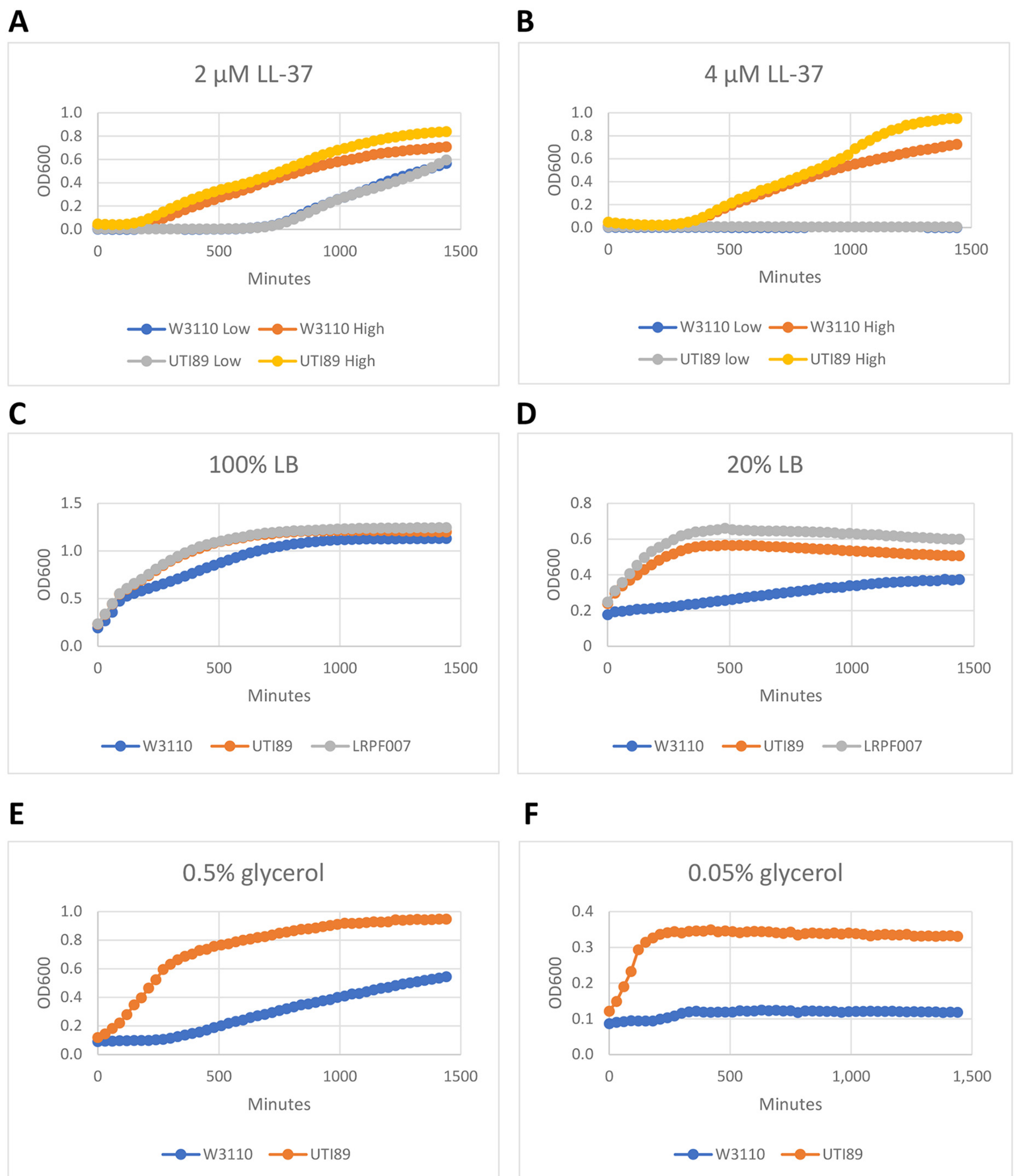

FIG 2 Effect of the antimicrobial peptide LL-37 and media dilution on UTI89, LRPF007, and W3110. Growth with low and high inoculation densities with 2 μM LL-37 (A) and 4 μM LL-37 (B). Growth in 100% LB (C) and 20% LB (D). Growth in a minimal medium with 0.5% glycerol as sole carbon source (E) and 0.05% glycerol as sole carbon source (F).

and B) but is less apparent at $\leq$1.3 μM (not shown). Because of this effect, we analyzed growth in all urine samples after a low- and high-density inoculation as an indirect test for urinary IFs. We define low- and high-density inoculations as initial OD600s of 0.004 and 0.02, respectively, and the final growth ratio after a high versus low inoculation density as the HL ratio. For W3110, the HL ratios were high and variable which suggests sensitivity to IFs and substantial IF variation between individual urine samples (Table S1). For the UPEC strains, the average HL ratios were near 1.0 regardless of patient group (Table S1), which implies resistance to the IFs in the urine samples and that UPEC growth is a measure of utilizable nutrient

content. The comparative growths of the two UPEC strains in current, never, and history group urine samples had ratios of approximately 3 to 2 to 1 (Table S2). For the current versus history groups, which has the highest ratio, the results achieve statistical significance for the high-density inoculations but not the low-density inoculations. In the nutrient-poor history group urine samples, both UPEC strains grew at least 2-fold better than W3110 (not shown). The UPEC strains also grew better than W3110 in diluted minimal and rich media (Fig. 2D to F), which shows that the UPEC strains utilize low levels of nutrients better than W3110.

Urine collection and processing. Following IRB approval from both University of Texas Southwestern Medical School and University of Texas at Dallas, midstream urine samples were collected from postmenopausal women who had not been on antibiotics for at least 4 weeks. The urine was frozen at −80C. For the growth experiments, urine was thawed, centrifuged to remove particulates, and sterilized with a 0.2 μm filter. Each well in a microtiter dish received 0.2 mL urine.

Bacterial strains. W3110 is a commonly used nonpathogenic lab strain. UTI89 is a model UPEC strain (11). LRPF007 is a UPEC strain from a patient with RUTIs which was isolated after plating on ChromeAgar, streaked on L-broth to obtain single colonies, and cells from a single colony were grown in L-broth. Glycerol was added to the culture which was frozen at -80 C. The strain was determined to be *E. coli* and phylotyped as described (13).

Bacterial growth. Bacterial strains were streaked on an L-broth agar plate and grown overnight. A single colony was inoculated into 1 mL L-broth for 2 h in a 15 mL glass tubes at 250 rpm, centrifuged and resuspended in PBS three times to remove residual medium, resuspended in PBS, and then diluted into urine samples. Bacteria were grown in triplicate wells and optical density at 600 nm was measured in BioTek plate readers. The ΔOD600s ranged from 0.023 to 0.424. For cultures with a ΔOD600 $>$ 0.1, the initial doubling time varied from 51 to 207 min and did not correlate with the ΔOD600. For the ΔOD600, the average coefficient of variation (the ratio of the standard deviation to the average of the triplicate determinations) was 13%.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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