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Role of Bacterial Growth Rates in the Epidemiology and Pathogenesis of Urinary Infections in Women

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The mean minimum generation time in shake culture in urine of 6 urinary isolates of *Escherichia coli* (21.7 ± 0.6 min) was significantly shorter ($P = 0.0003$) than that of 14 isolates of less common urinary pathogens (46.0 ± 18.6 min). Mixed populations of approximately equal numbers of *E. coli* cells paired with other urinary, fecal, and urethral organisms were introduced into a laboratory model of the lower human urinary tract. This model used urine as a medium and reproduced some features of the balance between bacterial growth and the flushing effect of urine. After 24 h *E. coli* formed ≥99% of the bacterial population in the bladder model for 16 out of 18 pairs of isolates examined. Relatively high oxygen tensions in urine samples from 18 healthy women (10.9 ± 2.2 kPa) and 18 infected patients (8.0 ± 4.3 kPa) may explain why anaerobic urinary infections are uncommon. The rapid growth rate of *E. coli* may be one explanation why it is the commonest cause of urinary infection even though it is relatively uncommon at the urethral meatus.

A mixture of organisms present in the periurethral area may be introduced into the bladder at intercourse (2), at catheterization (4), and perhaps by other means. However, acute urinary infections are usually due to a single pathogen. Moreover, organisms commonly causing acute urinary infections are not those most prevalent at the periurethral area. We have tested the hypothesis that differential growth rates in urine may be a selective factor allowing such organisms as *Escherichia coli* to more commonly cause recognizable infection.

Growth properties of a variety of aerobic and anaerobic bacteria in pure culture in urine in shake flasks were consistent with this hypothesis. Mixed cultures of *E. coli* paired with other organisms were incubated in urine in a model which reproduced some physiological features of the human urinary tract. Under these conditions *E. coli* outgrew other organisms in most instances. Oxygen tensions were measured in urine samples from healthy volunteers and infected patients and during in vitro studies. Evidence was obtained that oxygen levels in vivo are high enough to inhibit growth of strict anaerobes in urine.

MATERIALS AND METHODS

Organisms. Twenty aerobic isolates, listed in Tables 1 and 4, were obtained from general practice patients and hospital outpatients with significant urinary infections (≥10^5 organisms per ml in a single midstream specimen). Six aerobic cultures, listed in Table 2, were obtained from hospital inpatients from sites other than the urinary tract. Strict anaerobes in Tables 2 and 4 were obtained from the following sources: *Bacteroides fragilis*, perineal swab of a healthy woman and a pelvic wound; *B. melaninogenicus*, urethral swab of a healthy woman and a brain abscess; and *Clostridium perfringens*, feces from a healthy woman and a pelvic wound. *Enterobacteriaceae* were identified by the API 20E sysetem (API Laboratory Products Ltd., St. Laurent, Quebec, Canada) or by other conventional methods (3). The identity of *Staphylococcus saprophyticus* biotype 3 was confirmed by the novobiocin sensitivity test (8). Anaerobes were identified by John Ngui-Yen of Vancouver General Hospital (Vancouver, British Columbia, Canada) by the API 20A system.

Determination of bacterial growth rates in shake culture. Urine was sterilized by filtration, and an initial inoculum of 5×10^3 to 50×10^3 organisms per ml was used. Bacterial populations were determined at timed intervals in shake culture in urine. Parallel experiments were carried out in nutrient broth (BBL, Microbiology Systems, Cockeysville, Md.). Full experimental details have been described previously (1).

Bladder model. The bladder model (Fig. 1) was an apparatus fitted together with ground glass and polytetrafluoroethylene joints which facilitated steam sterilization. A cylindrical glass vessel (volume, 500 ml) served as the culture chamber. The lower part tapered, and interchangeable glass bases could be fitted to vary the residual volume. A residual volume of 1 ± 0.2 ml

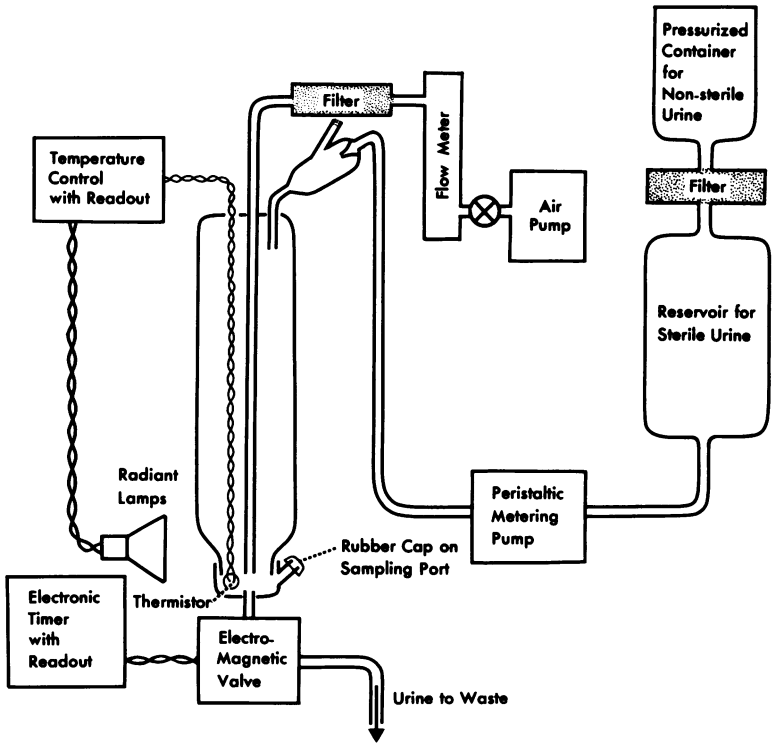

FIG. 1. *Flow diagram to illustrate the main features of a continuous-culture model of the human lower urinary tract.*

was chosen for all experiments described here. This value was determined by filling the apparatus with a solution of methylene blue which was drained and replaced with water (200 ml). The residual volume was calculated from observed changes in dye concentration. Viable counts were determined and oxygen tensions were monitored by withdrawing samples with a hypodermic syringe and needle via a rubber cap in the base of the apparatus. Cultures were aerated and mixed by passing air (750 ml/min) through the urine. The apparatus was maintained at a temperature of 37°C by three photoflood lights controlled by a thermostat activated by a thermistor in the base of the apparatus. Once the contents of the chamber reached 37°C, current through the lamps was reduced to control the temperature and minimize the effects of strong light. The "bladder" emptied via a sterilizable electromagnetic valve (model 800-1132; Mace Corp., South El Monte, Calif.) activated every 4 h by a timing device. Urine was pumped into the bladder through a peristaltic metering pump (P3; Pharmacia Fine Chemicals, Uppsala, Sweden). An urine flow rate of 50 ml/h was used.

Operation of the bladder model. Pooled midstream urine samples from males and females was cooled (4°C) immediately after voiding and sterilized by filtration through a 14-cm asbestos-cellulose filter (Carlson-Ford HP/EKS; General Filtration and Engineering Ltd., Scarborough, Ontario, Canada) and then stored at room temperature for up to 36 h. Precipitates rarely formed under these conditions. Foaming was controlled by addition of sterile silicone antifoam (0.05 ml/liter; A5633; Sigma Chemical Co., St. Louis, Mo.).

Aerobic organisms from overnight broth cultures (4 ml) were centrifuged and then suspended in saline to a laboratory standard of turbidity known to correspond to 10^8 *E. coli* cells per ml. Standardized suspensions were diluted 1:1,000 in sterile urine. Anaerobic bacteria from 48-h cultures on blood agar plates were suspended in saline and treated in like manner. Equal volumes (5 ml) of appropriate urinary suspensions were mixed and introduced into the bladder model, which had been previously stabilized at 37°C. After mixing with residual urine (1 ml) in the apparatus, a sample was withdrawn for viable count determination. Further samples were obtained at the intervals shown in Table 4. Viable counts were determined by plating serial dilutions on solid media.

Media and growth conditions for quantitation of bacteria. Differential media were used to distinguish *E. coli* from other pathogens in mixed cultures. All media were based on Difco heart infusion agar (0044-01) containing 5% human blood or Difco MacConkey agar with crystal violet (0470-01). *Streptococcus faecalis* was enumerated on MacConkey agar containing nalidixic acid (10 μg/ml). *Serratia marcescens*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Proteus* spp. were all ampicillin resistant and grew on MacConkey agar containing ampicillin

(25 μg/ml). Both base and inhibitory media for *Proteus mirabilis* were supplemented with additional agar (1%, wt/vol) to prevent swarming. *S. saprophyticus* was grown on blood agar with nalidixic acid (10 μg/ml). *Staphylococcus epidermidis* grew on blood agar containing sodium azide (100 μg/ml). All anaerobic organisms were incubated anaerobically for 48 h on blood agar supplemented with menadione (10 μg/ml) and gentamicin sulfate (10 μg/ml).

Determination of urine oxygen tensions. One midstream specimen of urine was collected from each of 18 patients (mean age, 41.4 years; standard deviation, ±18.6 years) of general practitioners with urinary infections and from 18 healthy females (mean age, 35.8 ± 13.8 years). The age distributions of the two groups were not significantly different ($P = 0.31$). Immediately after voiding, urine was drawn into a syringe, and oxygen tensions were measured within 5 min on a Corning EEL model 165 blood gas analyzer. No appreciable change in oxygen tensions took place over at least 10 min. Urine bacterial counts were quantitatively determined in both groups.

Urinary oxygen tensions in in vitro studies were measured in a similar manner within 1 min of withdrawing a sample.

Statistical analysis of data. Most analyses were carried out by a two-tailed Student's *t* test, assuming normal distribution and equal variance. Comparisons of bacterial growth rates in shake culture and urinary oxygen tension in infected patients and controls assumed normal distribution only. The ranges of pH values in Table 5 have been presented as medians and quartiles, since these are logarithmic values. However, mean values were used to compare the significance of differences in pH between the two populations because the Student *t* test is sufficiently robust.

A 1:1 ratio of *E. coli* to second pathogen was intended as the inoculum in each experiment. Bacterial suspensions could not be accurately predicted by standardizing the turbidity of inocula, and results have been included in which the initial ratio varied over the range 0.5:1 to 2:1. For ease of comparison, ratios in Table 4 have been recalculated and based on an initial nominal ratio of 1.0:1.0.

Assessment of the effect of antifoam and filtration on bacterial growth. Specimens of urine were divided into two portions: one was filtered through a thick sterilizing filter with addition of antifoam, as for the bladder model; the other was filtered through a membrane filter (Millipore Corp., Bedford, Mass.; type HA, 0.45-μm pore size). Growth characteristics of a selection of organisms were determined in shake culture as described above, using urine filtered by both methods.

Effect of oxygen tension on growth of test strains. Maximum growth rates of urinary suspensions (20 ml) of a selection of organisms were determined in shake flasks (250 ml) gassed (100 ml/min) with air or an air-nitrogen (1:1.8, vol/vol) mixture. Provision was made for withdrawing samples from the flasks with a needle and syringe through a rubber cap. General details of the method have been described above. Anaerobes were also grown in parallel in flasks gassed with nitrogen alone.

Effects of selective media on bacterial counts. Bacterial suspensions (approximately 10^5 organisms per ml) in urine were quantitatively enumerated on noninhibitory media. Mixtures of organisms listed in Table 4 were prepared which contained *E. coli* and the second pathogen in ratios of 1:1, 100:1, and 1,000:1. Bacterial populations in these mixtures were enumerated on MacConkey and other appropriate selective media. In each case the selective medium was shown to prevent growth of *E. coli* without inhibiting the second pathogen.

RESULTS

Generation times of a selection of 10 urinary isolates (*S. faecalis*, *Providencia stuartii* [*Proteus inconstans*], *P. aeruginosa*, *S. marcescens*, *S. saprophyticus* biotype 3, and *Proteus* spp.) were between 15 and 70% longer in urine than in broth, in contrast to 6 isolates of *E. coli*, where values for urine and broth were similar. The use of urine as a medium for growth studies is thus justified. Growth properties of bacteria were measured in shake culture in urine to test the hypothesis that *E. coli* might be able to outgrow other organisms in urine. The mean generation time and lag period of 6 urinary isolates of *E. coli* were significantly different ($P = 0.0003$ in both cases) from the corresponding values for 14 other urinary pathogens (Table 1). Growth properties of aerobic organisms isolated from sites other than urine resembled those of their corresponding urinary isolates in shake culture (Table 2). All anaerobic organisms died under these experimental conditions (Table 2).

TABLE 1. *Comparison of growth properties of urinary isolates of E. coli and other urinary pathogens in shake culture in urine*

Isolate	Minimum generation time (min)	Lag period (min)
E. coli A	21.1	27
E. coli B	21.8	53
E. coli C	22.1	59
E. coli D	20.9	62
E. coli E	22.4	31
E. coli F	22.1	83
S. faecalis A	59.8	78
S. faecalis B	62.1	154
S. marcescens A	34.1	101
S. marcescens B	35.5	99
P. aeruginosa	50.2	88
P. mirabilis	39.2	71
Proteus rettgeri	37.6	121
Proteus vulgaris	26.8	89
P. stuartii A	45.5	107
P. stuartii B	27.1	130
S. saprophyticus biotype 3, A	56.5	145
S. saprophyticus biotype 3, B	54.3	129
K. pneumonia	22.4	49
S. epidermidis	92.7	131

TABLE 2. *Growth properties of organisms isolated from sites other than urine in shake culture in urine*

Isolate	Site of isolation	Minimum generation time (min)[a]	Lag period (min)
E. coli	Fecal	22.5	76
E. coli	Urethral introitus	21.0	70
P. mirabilis	Fecal	40.1	121
P. mirabilis	Perineum	38.0	75
S. epidermidis	Perineum	82.0	131
S. faecalis	Perineum	57.0	111
C. perfringens	Feces	−32.9	
C. perfringens	Pelvic wound	−15.0	
B. melaninogenicus	Distal urethra	−7.9	
B. melaninogenicus	Brain abscess	−10.0	
B. fragilis	Perineum	−1220	
B. fragilis	Pelvic wound	−1040	

[a] Negative values denoting maximum death rates are approximations, because the death rate curves did not follow first-order kinetics closely.

TABLE 3. *Effect of method of sterilization on the growth-supporting properties of five samples of urine for 15 aerobic isolates in shake culture*

Urine treatment method	Mean (± standard deviation)		
	Lag period (h)	Minimum generation time (min)	Population after incubation for 24 h (per ml)
A. Millipore sterilization	2.14 (±1.48)	35.9 (±17.5)	9.54×10^8 ($\pm 7.74 \times 10^8$)
B. Carlson-Ford filter pads after addition of silicone antifoam	2.13 (±1.45)	35.1 (±17.0)	8.92×10^8 ($\pm 7.09 \times 10^8$)
Significance of difference of A versus B: P by two-tailed Student's t test	0.999	0.897	0.861

These experiments were then extended to more realistic test conditions in a model of the human lower urinary tract. In this model silicone antifoam was added to urine which was sterilized by filtration through thick asbestos-cellulose sterilizing pads. These filters absorb dyes, such as methylene blue, and might therefore affect the growth-supporting properties of urine. One portion of each of five specimens of urine was therefore filtered through a nitrocellulose membrane, which is unlikely to remove soluble constituents; the other was filtered through an asbestos-cellulose sterilizing pad with addition of antifoam. The growth characteristics of a selection of five *E. coli* isolates and one of each other aerobic pathogen listed in Table 4 were shown to be unaffected by antifoam or asbestos-cellulose filters (Table 3). All anaerobic organisms listed in Table 2 died under aerobic conditions in urine filtered by both methods. Oxygen tensions in the bladder model were usually 16 kPa and seldom fell below 8 kPa, in contrast to the human bladder (see below and Table 5). This potential artifact is probably unimportant, because minimum generation times of a selection of five isolates (as for Table 3) were similar in shake culture in the presence of air (≥18 kPa) and in the presence of a nitrogen-air mixture yielding an oxygen tension of 7.5 ± 1.0 kPa (mean minimum generation times were: 39.5 ± 21.6 and 40.8 ± 22.2 min, respectively). None of the six anaerobes listed in Tables 2 and 4 grew in the presence of the nitrogen-air mixture. Clostridia and *B. fragilis* listed in Table 2 grew anaerobically in urine, whereas *B. melaninogenicus* isolates died.

Mixed populations of *E. coli* with other aerobic and anaerobic organisms were studied in this bladder model. In each experiment approximately equal numbers of *E. coli* cells and cells of a second isolate were inoculated into the apparatus, and changes in bacterial populations were monitored. The use of one of the urinary isolates of *E. coli* as a reference standard in each experiment helped compensate for variations in the growth-supporting properties of different batches of urine. *E. coli* outgrew almost all other pathogens in mixed culture (Table 4). The mean oxygen tension in infected patients was slightly but significantly lower ($P = 0.01$) than that in healthy controls (Table 5). The distribution of values differed; the lowest oxygen tension in the control group was 8.19 kPa, whereas the three lowest in the infected patient group were 1.28, 1.90, and 5.30 kPa. There was an inverse relationship between oxygen tension and bacterial population in infected patients (correlation coefficient = 0.47; $P = 0.05$). Differences in urinary pH between the two groups were not significant ($P > 0.05$). Urinary bacterial and leukocyte counts in the two groups were clearly different.

DISCUSSION

E. coli is the commonest cause of urinary infection, yet it is relatively uncommon in the area of the urethral orifice (6, 9). Results with pure cultures in shake flasks and mixed populations in the bladder model support the hypothesis that differential growth rates in urine will tend to select this organism in pure culture. Other organisms may have pathogenic propensities which may be advantageous in certain

TABLE 4. *Comparison of the relative growth rates of E. coli and other isolates in mixed culture in a model of the human bladder*

Pair of isolates inoculated into bladder			Ratio[a] of population of second urinary pathogen/population of reference *E. coli* in urine sampled from the bladder model at:			
E. coli reference strain (urinary)	Second isolate (origin)		2 h	4 h	6 h	24 h
A	*S. faecalis* A	(urinary)	0.8	0.9	1.2	0.02
B	*S. faecalis* B	(urinary)	1.1	0.4	0.4	0.009
B	*S. marcescens* A	(urinary)	0.7	0.1	0.3	0.005
C	*S. marcescens* B	(urinary)	0.8	0.2	0.4	0.003
A	*P. aeruginosa*	(urinary)	0.7	0.4	0.1	0.01
B	*P. mirabilis*	(urinary)	1.3	0.08	0.03	0.002
B	*P. rettgeri*	(urinary)	0.2	0.01	0.003	0.02
B	*P. vulgaris*	(urinary)	0.4	0.09	0.02	0.002
D	*P. stuartii* A	(urinary)	3.3	0.03	0.03	0.03
D	*P. stuartii* B	(urinary)	0.1	0.01	0.01	0.0002
E	*S. saprophyticus* biotype 3, A	(urinary)	0.2	0.008	0.006	0.000002
D	*S. saprophyticus* biotype 3, B	(urinary)	0.4	0.04	0.008	0.000005
F	*K. pneumoniae*	(urinary)	2.7	4.6	2.7	0.6
F	*S. epidermidis*	(urinary)	0.6	0.3	0.001	0.00008
A	*B. fragilis*	(fecal)	0.2	0.007	0.0001	0
B	*B. melaninogenicus*	(urethral)	0	0	0	0
A	*C. perfringens*	(fecal)	0.7	0.0004	0.000003	0

[a] See the text section for method of calculation. Nominal ratio at the start of each experiment = 1.0, 0 denotes values of <0.000001.

TABLE 5. *Comparison of properties of urine from two groups of females*

Group	Mean urine bacterial count/ml (± standard deviation)	Median[a] urine leukocyte count/mm^3	Median[a] pH	Mean oxygen tension (kPa) (± standard deviation)
18 patients with significant urinary infection	6.4×10^5 ($\pm 1.3 \times 10^6$)	560 (44, 960)	5.50 (5.35, 5.61)	8.0 (±4.3)
18 healthy controls	9.1×10^2 ($\pm 2.0 \times 10^3$)	0 (0, 10)	5.89 (5.35, 6.40)	10.9 (±2.2)

[a] Median was used instead of mean because of wide distribution of leukocyte counts in infected patients and because pH is a logarithmic scale (quartiles in parentheses).

circumstances. For example, *Pseudomonas* and *Serratia* are seldom found except in catheterized patients or those with gross abnormalities of the urinary tract. Similarly, *S. saprophyticus* biotype 3 is largely limited to women between 16 and 25 years of age (7).

The artificial bladder apparatus is a crude model of the lower urinary tract which simulates conditions in the early stage of infection before the occurrence of tissue colonization and upper tract involvement. A shortcoming of the model was that no provision was made for sterilizing the urine drain tube to the solenoid valve during experiments. Furthermore, spray containing organisms swept into the upper part of the apparatus might slowly return to contaminate later samples. Such contamination will be particularly relevant with slow-growing organisms, where the population ratio in mixed culture would be expected to fall to very low values. For example, for the first pair of isolates in Table 4 the calculated ratio of *S. faecalis* to *E. coli* would be 2.5×10^{-12} at 24 h if one applied observed generation times and lag periods. Despite this reservation, most cultures from the artificial bladder at 24 h would be reported as a significant growth of a single strain if submitted as midstream specimens of urine to conventional procedures in a routine clinical laboratory.

In contrast to *E. coli*, obligate anaerobes, which seldom cause urinary infection (5, 10), are frequently found at the urethral meatus of healthy females (6). Oxygen tensions found in urine of all 18 healthy women in this study are likely to prevent multiplication of those anaerobes which would otherwise grow in urine. Shake flask and bladder model experiments provided support for this view. Although complete

anaerobiosis was not observed in any individual, relatively low values were found in some patients with aerobic infections. There is a possibility that dense cultures of aerobes might consume sufficient oxygen to permit growth of anaerobes. Also, as in the mouth, colonization of epithelia by dense cultures of bacteria could permit establishment of oxygen gradients and conditions suitable for strict anaerobes. Similar urinary pH values in infected and noninfected patients suggest that hydrogen ion concentration change is not a factor influencing differential bacterial growth in vivo.

Antibacterial properties of the urothelium, leukocytes, tissue infection, frequency of colonization or contamination of the distal urethra, and virulence attributes of individual bacteria are all factors which help determine which organisms are able to establish infection. Relative growth rates in urine appear, however, to be a major factor in explaining the observed frequency distribution of urinary pathogens.

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