THE RELEVANCE OF GROWTH RATES IN URINE TO THE PATHOGENESIS OF URINARY-TRACT INFECTIONS DUE TO *MICROCOCOCCUS* SUBGROUP 3 (*STAPHYLOCOCCUS SAPROPHYTICUS* BIOTYPE 3)

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SOME subgroup-3 micrococci, characterised by their resistance to novobiocin, differ from other Micrococcaceae in being primary urinary-tract pathogens. Following the original observations of Pereira (1962) and Mitchell (1964), this virulent biotype has been found to be the second commonest cause, after *Escherichia coli*, of acute urinary infection in otherwise healthy young women. Maskell (1974) has reviewed the earlier literature (see also Meers, Whyte and Sandys, 1975; Sellin *et al.*, 1975; Telander and Wallmark, 1975). This micrococcus biotype produces symptoms and pyuria comparable in severity to those produced by *E. coli*, and it appears to have a selective pathogenicity for the urinary tract of young women.

The classification of the Micrococcaceae has undergone several changes. Baird-Parker (1963) separated the genera *Staphylococcus* and *Micrococcus* by the Hugh-Leifson test, which determines the ability of bacteria to ferment or oxidise glucose. These genera were further divided into types by other biochemical tests. Errors in interpreting the Hugh-Leifson test sometimes arose from the poor anaerobic growth of some organisms. Recently, changes in classification have been made which depend upon DNA base composition and on cell-wall structure, demonstrated by lysis with lysostaphin. As a result, *Micrococcus* subgroups 1, 2, 3 and 4 were transferred to the species *Staphylococcus saprophyticus* (Baird-Parker, 1974). The precise taxonomic category of these organisms is still uncertain, but it is likely that only *Micrococcus* subgroup 3 will be included in *S. saprophyticus* (Dr A. C. Baird-Parker, personal communication). To obtain consistency with recent literature, the original Baird-Parker (1963) classification has been retained for this publication.

O'Grady *et al.* (1968) pointed out that the defences of the urinary tract operate at two levels: hydrokinetic mechanisms are concerned with the dilution and bulk removal of infected urine, and humoral and cellular mechanisms are concerned with the elimination of tissue infection. There is a possibility that the relative virulence of some subgroup-3 micrococci might be due to an enhanced ability to grow in urine from females. We have investigated this possibility by comparing the growth characteristics of novobiocin-resistant

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subgroup-3 micrococci with other Micrococcaceae and *Escherichia coli* in shake culture in urine from females.

MATERIALS AND METHODS

Subjects. Urine for most experiments was obtained from three healthy women aged 23, 24 and 27 years. One of these volunteers had had a urinary infection caused by a coliform bacillus 1 year previously. The others had never had urinary-tract infections. For certain experiments, urine was obtained from women aged 19, 23 and 30 years who had had urinary infections of novobiocin-resistant subgroup-3 micrococci within the previous 9 to 11 months.

Preparation of urine for growth studies. To determine whether filtration altered the nutritive properties of urine, the growth characteristics of five subgroup-3 micrococci and five strains of *Escherichia coli* were determined, as described below, in filtered and unfiltered midstream urine from males and in filtered midstream urine from females. Urine from women was usually too heavily contaminated for use without filtration. In these preliminary experiments, the generation time and other growth characteristics of the cocci (and also of coliform bacilli) were found to be similar in urine from males and females, whether filtered or not.

Experiments to compare the growth characteristics of different organisms in urine from females were performed with forestream (first 10–15 ml) and midstream urine from the volunteers. After voiding, the specimens were immediately cooled to 4°C. At the end of each day, a pool of forestream specimens and a pool of midstream specimens were centrifuged at 1500 *g* for 5 min. to remove mucus that might otherwise have blocked the filters. The supernates were then sterilised by filtration and used for experiments on the next day.

Organisms. Ten isolates of *E. coli* and three of novobiocin-resistant subgroup-3 micrococci were obtained from the urine of female general-practice patients in York with acute urinary infections. A further seven similar micrococci had been isolated in Bristol from the urine of female students with acute infections (Sellin *et al.*, 1975).

Twenty-one Micrococcaceae were selected from the organisms isolated from the urethral flora of healthy female students and from peri-urethral swabs taken at a family-planning clinic (Sellin *et al.*, 1975). These organisms were identified as *Micrococcus* 1, (one); *Micrococcus* 2, (four); novobiocin-resistant *Micrococcus* 3, (five); novobiocin-sensitive *Micrococcus* 3, (two); *Staphylococcus* ii, (eight); *Staphylococcus* v, (one). Methods of identifying Micrococcaceae were based on those described by Baird-Parker (1963) and have already been defined (Sellin *et al.*, 1975).

Growth-rate experiments. Urine (25 ml) in sterile 150-ml flasks was pre-warmed on an orbital shaker at 37°C and inoculated with a concentrated, saline-washed suspension of overnight broth or urine cultures to give cell densities of *c.* 10⁴ viable organisms per ml. Samples of urine were withdrawn immediately after inoculation and at intervals of 2 h up to 12 h and again at 24 h, for viable count determinations (Miles, Misra and Irwin, 1938) on nutrient-agar medium (Columbia Agar, Oxoid). Intervals between sampling times were varied in some experiments.

The growth of each culture was plotted on semi-logarithmic graph paper to determine the phase of exponential growth. The generation time was then calculated by performing linear regression analysis by the method of least squares on data obtained during the period of exponential growth. Cultures that had been incubated for 24 h were almost all in the stationary phase of growth. The intercept of the initial viable count with the calculated regression line (for the exponential portion of the plot of log viable count versus time) was regarded as the lag phase for the purposes of these experiments. The significance of differences between the mean values of growth parameters was determined by the two-tailed Welch test (Aspin, 1949, cited by Mack, 1967; Trickett, Welch and James, 1956, cited by Mack, 1967).

Growth rates in mixed culture. Urine was seeded with individual or mixed inocula and incubated as described above. In each experiment a novobiocin-resistant subgroup-3 micrococcus was paired with a novobiocin-sensitive micrococcus or staphylococcus. Viable counts were determined on both Columbia agar and Columbia agar containing novobiocin

TABLE I
Growth of virulent and non-virulent Micrococcaceae and of Escherichia coli in broth and in urine

Test designated	Organisms	Growth medium	Mean generation time (min.) during exponential growth	Mean lag period (h)	Mean viable count per ml (10^7) after 24 h
A	Ten novobiocin-resistant subgroup-3 micrococci from urinary infections	Pooled urine from three healthy young women	63.8 (21: $\pm$ 19.7)	4.7 (21: $\pm$ 1.2)	7.4 (21: $\pm$ 4.0)
B	Same as for test A	Nutrient broth	33.1 (10: $\pm$ 5.4) <0.01	1.9 (10: $\pm$ 1.0) <0.01	92.0 (10: $\pm$ 5.2) <0.01
C	Ten <i>E. coli</i> isolates from urinary infections	Same as for test A	22.4 (22: $\pm$ 3.6) <0.01	1.6 (22: $\pm$ 0.5) <0.01	131.0 (22: $\pm$ 103) <0.01
D	Five novobiocin-resistant subgroup-3 micrococci from the perineal-urethral flora of healthy women	Same as for test A	54.4 (8: $\pm$ 17.5) >0.10	4.7 (8: $\pm$ 1.5) >0.10	7.2 (8: $\pm$ 8.1) >0.10
E	Sixteen other Micrococcaceae from the perineal-urethral flora of healthy women	Same as for test A	44.8 (25: $\pm$ 12.5) <0.01	3.0 (25: $\pm$ 0.9) <0.01	18.0 (25: $\pm$ 15.5) <0.01
F	Same as for test A	Pooled urine from three young women who had had recent infections of subgroup-3 micrococci	66.6 (10: $\pm$ 54.3) >0.10	4.5 (10: $\pm$ 3.3) >0.10	18.8 (10: $\pm$ 37.0) >0.10

Figures in brackets refer to the number of experiments performed and the standard deviation of the mean.

Figures in bold type indicate the significance of the difference (two-tailed Welch test) between the entry and the corresponding entry in the test designated A.

TABLE II

Comparisons of growth-supporting properties of midstream and forestream urine for eight novobiocin-resistant subgroup-3 micrococci

Type of urine	Mean generation time (min.) in exponential phase of growth	Mean lag phase (h)	Mean viable cell count per ml (10^7) after incubation for 24 h
Forestream	48.1 (± 14.3)	3.93 (± 1.66)	14.4 (± 14.8)
Midstream	49.7 (± 9.78)	5.02 (± 1.49)	5.28 (± 3.80)

Standard deviations are given in brackets.

(1.5 μ g per ml) at the start of each experiment and after 6 h. The selective medium was prepared by adding the novobiocin (Upjohn Company Ltd) from a sterile stock solution, to sterile agar that had been cooled to 45°C.

RESULTS

The mean generation times during exponential growth of ten novobiocin-resistant subgroup-3 micrococci from urinary infections were significantly longer in urine than in broth. The final viable count was significantly lower and the lag phase significantly longer in urine than in broth (table I, tests A and B). Similar differences were found between the growth-supporting properties of urine and of nutrient broth with the other Micrococcaceae. The use of nutrient broth for studies of the Micrococcaceae in relation to the urinary tract would therefore not be justified.

The novobiocin-resistant subgroup-3 micrococci that had caused urinary infections grew more slowly, had longer lag periods and reached lower final viable counts than did the *Escherichia coli* isolates (table I, tests A and C). The novobiocin-resistant subgroup-3 micrococci isolated from the urethra and vulva of healthy women did not differ significantly from those isolated from infected urine (table I, tests A and D). Surprisingly, the "non-pathogenic" Micrococcaceae isolated from healthy women grew significantly more quickly than did the virulent biotype and reached a higher final viable count (table I, tests A and E). Urine from women who had recently suffered an infection with the virulent biotype had similar growth-supporting properties to those of normal urine (table I, tests A and F). The enhanced virulence of subgroup-3 micrococci over other Micrococcaceae in the urinary tract of young women cannot, therefore, be due to rapid growth of this biotype in midstream urine.

To investigate the possibility that pathogenic biotype-3 micrococci have a predilection for the urethra, or that urethral secretions provide a growth factor, growth rates of eight novobiocin-resistant subgroup-3 micrococci were compared in forestream and midstream urine. Forestream urine would be expected to contain a higher proportion of urethral secretions. There was no significant

TABLE III

Effect of pre-incubation in urine on the growth characteristics in urine of seven novobiocin-resistant subgroup-3 micrococci

Inoculum grown overnight in	Mean generation time (min.) in exponential phase of growth	Mean lag phase (h)	Mean viable cell count per ml (10^7) at 24 h
broth	50.4 (± 10.1)	4.0 (± 1.5)	5.9 (± 0.8)
urine	49.8 (± 10.9)	2.7 (± 0.9)	6.7 (± 1.5)

Standard deviations are given in brackets.

difference between the growth rates or lag phases ($P > 0.10$), or final viable counts ($P > 0.05$) of organisms grown in urine from these two sources (table II). Another possibility to be considered was that contact with traces of urine on the perineum or urethra might enable the pathogenic micrococci to adapt to more rapid growth in urine in the bladder. However, pre-incubation in urine *in vitro* had no significant effect ($P > 0.05$ for each of the parameters described in table III).

To investigate the possibility that virulent micrococci interfere in some way with the growth of other micrococci, approximately equal numbers of overnight cultures of nine pairs of novobiocin-resistant subgroup-3 strains and other Micrococcaceae were inoculated into urine shake-cultures. No significant difference was found between the mean ratios of the viable counts of virulent and non-virulent strains after incubation for 6 h ($P > 0.10$).

DISCUSSION

The investigation showed that the micrococci that had caused urinary infection did not grow more rapidly than other, non-virulent Micrococcaceae. The virulent biotype must, therefore, possess a virulence attribute other than ability to grow rapidly in vesical urine. If infection was controlled solely by hydrokinetic factors, observed bacterial generation times could theoretically be used to calculate a range of conditions such as urine output, frequency, and residual volume, over which the virulent biotype might persist in the urinary tract (O'Grady and Cattell, 1966*a* and *b*). However, individual variations in urine output and frequency of micturition are quite large: for example, the daily frequency of micturition of 21 healthy young females in York ranged from 2 to 13 (mean 5.9 ± 2.2). Imperfect mixing, local underperfusion and other factors (discussed by O'Grady and Cattell, 1966*a*) make such quantitative predictions of limited value in our studies.

The presence of a "growth factor" in urethral secretion also seems unlikely, though our experiments did not exclude the possibility that such a factor might be associated with the mucus removed by centrifugation.

Novobiocin-resistant subgroup-3 micrococci are strongly urease positive, but so are a minority of the non-virulent Micrococcaceae isolated from the urinary tract (Sellin *et al.*, 1975). Although not a primary virulence determinant in micrococci, urease activity may play a contributory role in the pathogenesis of urinary infection, similar to that demonstrated with *Corynebacterium renale* and with *Proteus* spp. (Keppie, 1964).

A possible hypothesis, for which there is as yet no experimental evidence, is that the virulence of novobiocin-resistant subgroup-3 micrococci results from an ability to colonise and to attach to urethral or vesical epithelium.

SUMMARY

A novobiocin-resistant "biotype" of *Micrococcus* subgroup 3 (*Staphylococcus saprophyticus*) is known to be a primary pathogen of the female urinary tract and to cause infections as severe as those produced by *Escherichia coli*. The growth characteristics of this virulent biotype were compared *in vitro* with those of other Micrococcaceae and of *E. coli* to determine whether rapid growth explains the virulence of the biotype.

Nutrient broth was shown to have growth-supporting qualities that differed from those of urine and it was therefore unsuitable for these studies. In urine, the virulent biotype grew more slowly, had a longer lag period, and reached much lower final viable counts than did *Escherichia coli*. Surprisingly, the virulent biotype also grew more slowly and reached a lower final viable count than did several other Micrococcaceae isolated from the urinary tract of healthy women. Urine from women who had suffered a recent infection with the virulent biotype had growth-supporting properties similar to those of urine from healthy women.

Experiments with filtered forestream urine suggested that urethral secretions do not contain a factor determining growth rates of this organism in urine. The possibility that virulent strains adapt to rapid growth in urine was excluded. No evidence was obtained that the virulent biotype inhibits the growth of other Micrococcaceae in urine.

Ability to grow rapidly in urine does not therefore explain the virulence of novobiocin-resistant strains of subgroup-3 micrococci.

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REFERENCES

- BAIRD-PARKER, A. C. (1963). A classification of micrococci and staphylococci based on physiological and biochemical tests. *J. gen. Microbiol.*, **30**, 409.
- BAIRD-PARKER, A. C. 1974. Gram-positive cocci. In *Bergey's Manual of determinative bacteriology*, 8th ed., edited by R. E. Buchanan and N. E. Gibbons, Baltimore, p. 478.
- KEPPIE, J. 1964. Host and tissue specificity. In *Microbial behaviour "in vivo" and "in vitro"* (14th *Sympos. Soc. gen. Microbiol.*), edited by H. Smith and Joan Taylor, Cambridge, p. 44.

- MACK, C. 1967. Essentials of statistics for scientists and technologists, *New York*, pp. 48, 152, 154, 155.
- MASKELL, R. 1974. Importance of coagulase-negative staphylococci as pathogens in the urinary tract. *Lancet*, **1**, 1155.
- MEERS, P. D., WHYTE, W. AND SANDYS, G. 1975. Coagulase-negative staphylococci and micrococci in urinary tract infections. *J. clin. Path.*, **28**, 270.
- MILES, A. A., MISRA, S. S. AND IRWIN, J. O. 1938. The estimation of the bactericidal power of the blood. *J. Hyg., Camb.*, **38**, 732.
- MITCHELL, R. G. 1964. Urinary tract infections due to coagulase negative staphylococci. *J. clin. Path.*, **17**, 105.
- O'GRADY, F. AND CATTELL, W. R. 1966a. Kinetics of urinary tract infections. I. Upper urinary tract. *Br. J. Urol.*, **38**, 149.
- O'GRADY, F. AND CATTELL, W. R. 1966b. Kinetics of urinary tract infection. II. The bladder. *Br. J. Urol.*, **38**, 156.
- O'GRADY, F., GAUCI, C. L., WATSON, B. W. AND HAMMOND, B. 1968. In-vitro models simulating conditions of bacterial growth in the urinary tract. *In Urinary tract infection*, edited by F. O'Grady and W. Brumfitt, *London*, p. 80.
- PEREIRA, A. TORRES 1962. Coagulase negative strains of staphylococcus possessing antigen 51 as agents of urinary infection. *J. clin. Path.*, **15**, 252.
- SELLIN, M. A., COOKE, D. I., GILLESPIE, W. A., SYLVESTER, D. G. H. AND ANDERSON, J. D. 1975. Micrococcal urinary tract infections in young women. *Lancet*, **2**, 570.
- TELANDER, B. and WALLMARK, G. 1975. Micrococcus subgroup 3—a common cause of acute urinary tract infection in women. *Läkartidningen*, **72**, 1675.