Enhanced siderophore production and mouse kidney pathogenicity in *Escherichia coli* grown in urine

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Summary. Fifteen siderophore producing urinary isolates of *Escherichia coli* were compared for aerobactin and enterochelin production in trypticase soy broth and pooled normal human urine. Significant increase in siderophore production (both phenolate and hydroxamate) was observed when organisms were grown in urine. Mouse kidney pathogenic potential of the strains grown in urine was compared with that of bacteria grown in trypticase soy broth in an ascending model of pyelonephritis in female Swiss Webster mice. Organisms grown in urine and instilled into a mouse bladder demonstrated markedly enhanced renal pathogenicity ($p < 0.01$). Further information about the influence of urinary constituents on siderophore production could help in understanding the pathogenesis of pyelonephritis.

Introduction

With few exceptions, iron is essential for growth of bacteria.^{1–3} In spite of the abundance of iron in animal tissues, the amount available to the bacteria is extremely small.⁴ In the body, most of the iron is intracellular and extracellular iron is attached to high affinity iron binding glycoproteins.^{5,6} Under conditions of iron starvation, many micro-organisms release low-mol. wt compounds termed siderophores, which help them to sequester iron from the environment and from the host. In *Escherichia coli*, phenolates are synthesised by all strains^{7,8} but it has been reported that only some pathogenic strains elaborate aerobactin.⁹ The nature of the disease produced by a pathogen, i.e., localised or systemic, is influenced by its ability to sequester iron at different body sites.¹⁰ The importance of this kind of iron availability as a modulator of virulence of micro-organisms has received considerable experimental and speculative attention.^{11,12} The urinary tract provides an environment where iron is a limiting factor.¹³ This study examined how growth in normal urine *in vitro*, in a situation similar to that *in vivo*, affected the production of siderophores in *E. coli*. Furthermore, in order to assess its effect on overall virulence of the organism, a model of ascending pyelonephritis in mice was used to compare renal pathogenicity of organisms grown in urine and trypticase soy broth.

Materials and methods

Bacterial strains

Seventy urinary isolates of *E. coli* were examined for their ability to produce hydroxamate (aerobactin)

and 15 producer strains were selected for further study. The organisms were identified according to the methods of Brenner.¹⁴ The numbers of passages on agar were kept to a minimum before the study was performed. The strains were maintained as nutrient-agar stab cultures (pH 7.4) at 4°C.

Growth media

Filter-sterilised urine pooled from 3–4 human volunteers and Trypticase Soy Broth (TSB; Difco) were used as the growth media for cultivation of the *E. coli* strains. All the bacteria were grown overnight at 37°C without shaking. The organisms were passaged four times in urine. All the bacterial cultures were harvested by centrifugation. The supernates were collected for siderophore estimations. The bacterial pellets were washed three times with phosphate-buffered saline (PBS; pH 7.3, 0.01 M) and then suspended in PBS to give an optical density of 0.5 at 575 nm.

Siderophore estimation

For estimation of phenolates, the chemical assay of Arnow¹⁵ was used. The method of Gibson and Magrath¹⁶ was followed for the estimation of hydroxamates.

Mouse kidney pathogenicity

The ascending pyelonephritis model of Hagberg *et al.*¹⁷ was used but a slight modification, outlined by Sinha *et al.*,¹⁸ was added. Female LACA strains of Swiss Webster mice, which had sterile urine, were used and these animals were killed on the fifth day after infection. The kidneys were removed aseptically and examined bacteriologically and histopathologi-

cally. For bacteriological examination, one half of both kidneys was placed in sterile tubes, weighed and homogenised in a Corning glass homogeniser with 1 ml of sterile PBS. Serial dilutions of the homogenate were plated on MacConkey Agar, incubated at 37°C for 24 h, and the viable counts were determined from the colony counts.

For histopathological examination, the other half of kidney tissue, preserved in formalin, was dehydrated in an ethanol gradient (70–100%). The tissue was embedded in paraffin, then sectioned, stained and examined. The severity of infection was graded by the method of Garg *et al.*¹⁹

Statistical analysis

The F-test was used to assess the statistical significance between data from more than two groups and Student's *t* test was used to assess significant differences between two groups.

Results

Phenolate production by the 15 strains of *E. coli* after incubation for 24 h in TSB, and in normal human urine with up to four passages, is shown in table I. All the *E. coli* isolates produced small quantities of phenolates in TSB; these increased significantly ($p < 0.01$) when the organisms were grown in normal human urine (first passage). There were further increases with subsequent passages in urine. However, increased production in urine after the fourth passage was not significant ($p > 0.05$).

Hydroxamate production is shown in table II. In TSB, hydroxamate production was very low in most

strains tested. However, after they were grown in urine, there were significant increases in production of hydroxamate by all strains ($p < 0.01$). Further increases were observed with additional passages in urine. These increases were seen up to the third passage but were not evident after that.

The results in table III show the renal bacterial counts from mice when organisms had been instilled into the mouse bladder. There was a significant decrease in the numbers of organisms in the kidney when the organisms were initially grown in urine ($p < 0.05$). After the second passage in urine, there was a significant increase ($p < 0.05$) in mean renal viable counts compared with organisms used after the first passage. However, this increase was not significant in comparison to organisms grown in TSB ($p > 0.05$). The mean number of *E. coli* present in the kidney increased further only up to the third passage ($p < 0.01$) and no increase was found between the third and fourth passages ($p > 0.05$).

Histopathological examination of kidneys infected with randomly selected strains showed that, with strains grown in urine (third passage), pathogenicity in the ascending pyelonephritis model was enhanced (figs 1–4). The greatest inflammatory response was observed with strain no. 51 grown in urine, for which the average histopathological score was 11.8. The average score with strains grown in TSB was 2.16, and with organisms grown in urine, at the third passage, 9.4 (table IV).

Discussion

In this study, we found that all the urinary isolates, when grown in TSB, produced phenolates and that

Table I. Phenolate production by *E. coli* isolates in normal human urine and TSB

Strain no.	Optical Density at 515 nm of strains grown in				
	TSB (a)	urine			
		1st passage (b)	2nd passage (c)	3rd passage (d)	4th passage (e)
1	0.092	0.246	0.278	0.323	0.327
2	0.113	0.282	0.291	0.412	0.419
3	0.056	0.262	0.283	0.307	0.299
6	0.069	0.310	0.319	0.327	0.345
8	0.089	0.373	0.413	0.456	0.457
11	0.082	0.263	0.299	0.368	0.373
13	0.073	0.300	0.322	0.354	0.342
22	0.081	0.296	0.305	0.316	0.301
23	0.117	0.421	0.452	0.492	0.481
25	0.087	0.425	0.449	0.485	0.483
42	0.153	0.384	0.411	0.443	0.439
49	0.071	0.526	0.578	0.638	0.637
51	0.082	0.233	0.278	0.314	0.321
5	0.072	0.263	0.292	0.338	0.334
9	0.091	0.216	0.254	0.298	0.289

F-test, $p < 0.05$; Student's *t* test: a and b, $p < 0.01$, a and c, $p < 0.01$, a and d, $p < 0.01$, d and e, $p > 0.05$.

Table II. Hydroxamate production by *E. coli* isolates in normal human urine and TSB

Strain no.	Optical Density at 526 nm of strains grown in				
	TSB	urine			
		1st passage	2nd passage	3rd passage	4th passage
	(a)	(b)	(c)	(d)	(e)
1	0.031	0.276	0.498	0.613	0.609
2	0.011	0.198	0.356	0.425	0.427
3	0.019	0.278	0.443	0.512	0.517
6	0.014	0.162	0.419	0.523	0.522
8	0.012	0.167	0.307	0.329	0.329
11	0.017	0.097	0.286	0.315	0.327
13	0.032	0.268	0.278	0.342	0.351
22	0.026	0.220	0.429	0.612	0.615
23	0.032	0.079	0.413	0.478	0.462
25	0.015	0.109	0.293	0.367	0.366
42	0.021	0.168	0.377	0.527	0.528
49	0.017	0.214	0.421	0.568	0.558
51	0.026	0.155	0.418	0.537	0.539
5	0.013	0.125	0.357	0.498	0.492
9	0.016	0.235	0.378	0.532	0.531

F-test, $p < 0.05$; Student's *t* test: a and b, $p < 0.01$, a and c, $p < 0.01$, a and d, $p < 0.01$, d and e, $p > 0.05$.

Table III. Kidney colonisation *in vivo* by isolates of *E. coli* grown in normal human urine and TSB

Strain no.	Mean number of bacteria ($\log_{10}$ cfu/g) in kidney tissue of strains grown in				
	TSB	urine			
		1st passage	2nd passage	3rd passage	4th passage
	(a)	(b)	(c)	(d)	(e)
1	3.63	3.01	4.16	6.46	6.49
2	3.22	2.95	3.72	6.01	6.02
3	4.40	3.51	4.50	7.51	7.62
6	2.28	2.01	2.52	6.24	6.25
8	3.12	2.42	3.02	5.81	5.80
11	2.96	2.06	3.41	5.56	5.45
13	2.23	1.56	2.95	6.63	6.62
22	4.41	3.13	4.71	6.31	6.42
23	2.58	2.02	2.96	5.88	5.85
25	4.41	3.82	4.91	5.20	5.23
42	3.78	3.20	3.98	5.56	5.58
49	4.02	3.43	4.32	6.84	6.86
51	3.57	2.97	3.98	6.52	6.95
5	2.53	2.12	3.12	5.13	5.01
9	4.13	3.16	4.53	7.23	7.29

F-test; $p < 0.05$; Student's *t* test: a and b, $p < 0.05$, a and c, $p > 0.05$, b and c, $p < 0.05$, a and d, $p < 0.05$, c and d, $p < 0.01$, d and e, $p > 0.05$.

the amount of the phenolates produced increased up to the third passage in urine as a culture medium. Iron-deficient conditions in urine can explain these observations. Mutant strains of *E. coli* lacking ferric enterochelin esterase activity have been reported to be disadvantaged when grown in iron-deficient medium.²⁰ In relation to enterochelin production and virulence of *E. coli* it has been reported that iron-binding catechols are secreted in both synthetic

medium and in serum. Furthermore, transferrin and ethylenediamine-di-(*O*-hydroxyphenyl) acetic acid (EDDA) induce catechol production and abolish bacteriostasis *in vivo*.²¹

Williams and Carbonetti⁹ showed that although aerobactin has markedly lower affinity for iron than enterochelin, it provides a selective advantage for growth of bacteria in iron-limited conditions in body tissues and fluids. Aerobactin is repeatedly re-usable

Fig. 1. Photomicrograph showing histological appearance of normal mouse kidney (Severity score, 0); haematoxylin and eosin, $\times 100$.

Fig. 2. Photomicrograph showing dense perivascular infiltrate (Severity score, 2); haematoxylin and eosin, $\times 200$.

Fig. 3. Photomicrograph showing interstitial infiltrate consisting of polymorphs and lymphocytes (Severity score, 4); haematoxylin and eosin, $\times 200$.

Fig. 4. Photomicrograph showing multiple micro-abscesses in the renal cortex (Severity score, 12); haematoxylin and eosin, $\times 100$.

Table IV. Severity score of pathological lesions in kidneys infected with *E. coli* grown in TSB or urine

Strain no.	Mean (SD) Severity score with strains grown in	
	TSB	urine
3	1.80 (0.63)	10.80 (1.35)
13	2.00 (0.82)	7.50 (0.79)
25	2.17 (0.56)	7.80 (0.95)
51	2.68 (0.48)	11.80 (1.20)

For TSB *vs* urine, $p < 0.01$ (Student's *t* test).

and has been found to stimulate bacterial growth at extracellular concentrations 500-fold lower than that of enterochelin. Stuart *et al.*²² reported that the ability for hydroxamate-mediated transport of iron is widely distributed among natural isolates of *E. coli*, but that the distribution of hydroxamate-positive *E. coli* is not random. *E. coli* isolates from sources where levels of available iron are expected to be low tend to be hydroxamate-positive. Our present observations support the hypothesis that natural iron-limiting conditions, as prevail in urine, enhance the production of

hydroxamates. Whether enhanced elaboration of siderophores by organisms grown in urine depend on selection of phenotypic variation has yet to be explored. Bacterial counts in kidneys and observed inflammatory responses further substantiate the view that the overall virulence of organisms grown in urine is enhanced up to the third passage. Urine is a complex product, with variable amounts of uromucoid, T-H proteins and other chemicals. Their precise role, alone or in combination, in the elaboration of siderophores and virulence is currently being investigated further.

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