Characteristics of cysteine-requiring strains of *Klebsiella* isolated from urinary tract infections

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Summary. Clinical and bacteriological findings in seven cases of urinary tract infection with cysteine-requiring strains of *Klebsiella* are described. The organisms were isolated from patients with long-standing urinary tract abnormalities and grew as small (c. 1 mm) colonies on MacConkey agar. The organisms failed to grow in a minimal medium supplemented with sodium sulphate but grew when the medium was supplemented with cysteine sulphinic acid, sodium sulphide or L-cysteine. The smallest amount of cysteine required for optimal growth in a chemically defined medium was 20 mg/L. Cysteine-requiring strains of *Escherichia coli* had previously been shown to require a similar amount of cysteine and to be unable to reduce sulphate to sulphite; this suggests a common influence in the selection of cysteine auxotrophs *in vivo*. However, the amino acid inhibited the growth of *E. coli* at concentrations which only slightly altered growth of the *Klebsiella* strains. Problems with the isolation, identification and sensitivity testing of cysteine-requiring *Klebsiella* were also observed and methods by which these may be minimised are suggested.

Introduction

Enterobacteriaceae with a variety of metabolic defects are sometimes isolated from patients with urinary tract infections. These strains are most often seen in the elderly who have chronic diseases, in patients with structural abnormalities of the urinary tract (Gillespie, 1952; Borderon and Horodniceanu, 1978) or in those who have received co-trimoxazole therapy (Tapsall *et al.*, 1974).

Gillespie (1952) isolated and characterised strains with defects in sulphur metabolism that could be corrected, at least partially, by the provision of exogenous cysteine and other sulphur-containing compounds. Most of these urinary isolates were *Escherichia coli* and similar organisms have been recovered subsequently from blood cultures and wound abscesses (Tapsall and McIver, 1986). Gillespie (1952) also described one strain of "*Bact. aerogenes*" in his study of cysteine-requiring urinary isolates.

Although cystine-supplemented media are available to facilitate the isolation of these organisms (Mackey and Sandys, 1966; Bevis, 1968), a number of problems still exist with regard to the isolation, identification and susceptibility testing of cysteine-

requiring *E. coli*. We report seven cases of urinary tract infection caused by cysteine-requiring klebsiellae and discuss difficulties peculiar to their isolation and characterisation. Experiments designed to define further the metabolic defects, and the requirement for cysteine of these strains, are also described. Additionally, the toxic effects of higher concentrations of this amino acid were examined and the results compared with those previously obtained with auxotrophic *E. coli* under similar conditions (McIver and Tapsall, 1987).

Materials and methods

Bacteriological methods

The organisms were isolated from urine on MacConkey agar (Oxoid) and growth quantified by the filter paper method of Leigh and Williams (1964). The requirement for cysteine was shown on Sensitest Agar (Oxoid) by demonstrating growth only around a paper disk saturated with 0.63 mM L-cysteine hydrochloride (Sigma) (Tapsall and McIver, 1986). The isolates were identified by the methods of Cowan (1974) with conventional bacteriological identification media (Oxoid or Difco) and by the Analytical Profile Index 20E (API systems, La Balme les Grottes, France). Antibiotic susceptibility tests were performed by a modification of the calibrated dichotomous sensitivity (CDS) method (Bell, 1975) on Sensitest

Agar. The inocula were prepared in physiological saline (sensitivity and conventional identification tests) or sterile water (API system) with and without a supplement of L-cysteine HCl 100 mg/L (0.63 mM).

The phenotypes of the isolates were assigned according to their response to intermediates of the sulphate assimilatory pathway (Jones-Mortimer, 1968) by methods previously described for cysteine-requiring *E. coli* (Tapsall and McIver, 1986). Briefly, a suspension of the organism was prepared from an overnight culture in minimal medium (Pasternak, 1962) containing 0.63 mM L-cysteine HCl. After washing, this suspension was adjusted to 10^4 cfu/ml as determined by surface viable counts (Miles *et al.*, 1938). A volume of 0.025 ml of this suspension was then inoculated by means of a Steers-type replicating device on to minimal agar medium with and without sodium sulphate, cysteine sulphinic acid, sodium sulphide or L-cysteine HCl in concentrations previously described (Tapsall and McIver, 1986). The inoculated media were incubated aerobically for up to 3 days at 37°C and examined daily for growth.

The cysteine requirements of three auxotrophic strains were determined in a chemically defined medium (Davis and Mingioli, 1950) by methods previously described (McIver and Tapsall, 1987). Briefly, the organisms were grown overnight in defined broth supplemented with 0.63 mM L-cysteine HCl; the cultures were then centrifuged and washed three times in physiological saline and inoculated into 2.5 ml of defined broth to give an initial optical density (OD) at 640 nm of 0.05 in a Spectronic 20 spectrophotometer (Milton Roy, Rochester, NY, USA). The inoculated broths were incubated at 37°C in a shaking waterbath. The specific growth rates, length of lag phase and growth yield were calculated from growth curves constructed from OD readings taken at intervals of 30 min until the cultures reached stationary phase (Nagy *et al.*, 1969). The OD readings were related to

surface viable counts (Miles *et al.*, 1938). Solutions of cysteine were freshly prepared, filtered and added to the medium immediately before each experiment and the pH of all media was adjusted to 7.0 before use. All experiments were performed in duplicate and repeated twice. The prototrophic strain *Klebsiella pneumoniae* ATCC 12657 was used as a control.

Results

The clinical features of the seven patients with urinary tract infections caused by cysteine-requiring isolates of *Klebsiella* are shown in table I.

The seven isolates grew poorly on MacConkey agar, producing small mucoid colonies c. 1.0 mm in diameter. On medium supplemented with 0.63 mM cysteine, the colony size of the auxotrophs increased to about 3.0 mm in diameter and the typical morphological features of prototrophic strains of the genus were clearly recognisable. All strains were shown to be auxotrophic for cysteine by the paper disk method.

The organisms could not be identified by conventional techniques in the absence of cysteine supplementation. All seven strains failed to grow optimally in media used to test for lysine decarboxylase (Difco), for urease activity and for citrate utilisation (Oxoid). With the API 20E identification system, the strains failed to show a requirement for citrate and the Voges-Proskauer reactions were negative. Additionally, pH changes were slow to appear in the carbohydrate utilisation tests. Although some isolates were identified as "*Klebsiella* spp." in this

Table I. Clinical details of seven patients with urinary tract infections caused by cysteine-requiring *Klebsiella*

Patient	Age	Sex	Associated clinical features	Outcome
1	77	M	Bilateral hydronephrosis; prostatism; diabetes mellitus	Organism eliminated with clavulanate-potentiated amoxycillin
2	69	F	Recurrent UTI; renal calculi; analgesic nephropathy	Failed to settle on cephalexin therapy
3	83	F	Synovitis of knee; incontinence; residual urine after voiding	Not recorded
4	65	F	Renal transplant 14 years; immunosuppressed; diabetes mellitus	Responded to gentamicin therapy
5	28	F	Severe reflux nephropathy with scarring; recurrent UTI	Clinical pyelonephritis responded to co-trimoxazole therapy
6	74	M	Vesico-colic fistula and carcinoma of sigmoid; prostatism	Infection resolved with closure of fistula and transurethral resection of prostate
7	69	F	Analgesic nephropathy; un-united fracture of humerus	Died of unrelated causes. Granular, contracted kidneys at autopsy

UTI = Urinary tract infection.

system, four strains produced profile numbers which did not allow identification. Growth in both systems was enhanced when the inocula were prepared in solutions supplemented with 0.63 mM L-cysteine HCl. By this procedure, six of the seven auxotrophs were identified as *K. aerogenes* (*K. pneumoniae*—*sensu lato*) by the methods of Cowan (1974) and as *K. pneumoniae* by the API system. The other strain was identified by the methods of Cowan (1974) as *K. ozaenae*, and by the API profile number as an unspciated member of the genus *Klebsiella*.

None of the seven strains grew on Sensitest Agar when the inoculum was prepared in saline but when the saline suspensions were supplemented with cysteine HCl, the organisms grew well. The sensitivities of the organisms to six antimicrobial agents tested by the CDS method (Bell, 1975) are shown in table II. One strain was resistant to all six agents; additional tests showed it to be resistant also to tobramycin and kanamycin but sensitive to amikacin, netilmicin, cefotaxime and to the combination of amoxycillin and clavulanic acid.

All strains grew in minimal medium supplemented with cysteine sulphinic acid, sodium sulphide or cysteine, but not on unsupplemented medium or in the presence of sodium sulphate. The effects of increasing concentrations of cysteine on growth yield and specific growth rate are shown in the figure. The three auxotrophs tested each produced a similar growth curve in response to increasing levels of cysteine. The strains failed to grow in cysteine-deficient broth, but growth yields improved as the cysteine concentration was increased to 20 mg/L (0.13 mM). Above this concentration, up to a level of 1.27 mM cysteine (200 mg/L), similar growth yields were observed for each strain.

There were noticeable differences between the

auxotrophic strains and the prototrophic control with regard to the specific growth rate at cysteine concentrations in the range 20–300 mg/L (0.13–1.90 mM). With the auxotrophic strains there was a gradual increase in the specific growth rate as the concentration was increased, but there was no concomitant alteration in the growth yield. In contrast, the specific growth rate of the prototrophic control was similar at all concentrations of cysteine up to 300 mg/L, also with no marked changes in the growth yield. The specific growth rate and growth yield of all strains decreased slightly at concentrations of cysteine above 400 mg/L. The lag phase of all strains was slightly increased at cysteine concentrations in the range 20–300 mg/L but was reduced in the presence of higher levels of the amino acid.

Discussion

The only previous report of the isolation of cysteine-requiring *Klebsiella* was that of Gillespie (1952) who found a strain that “resembled *Bact. aerogenes*” in the urine of an elderly male patient. Only a limited amount of information on the strain was provided in this report, which dealt principally with cysteine-requiring *E. coli*. It was noted that auxotrophic *E. coli* were isolated most commonly from elderly patients with chronic renal tract problems. Six of the seven infections caused by cysteine-requiring klebsiellae which we report were also in elderly patients with chronic renal problems. One younger patient was aged 28 years at the time of her infection; she suffered from long-standing reflux nephropathy and renal scarring was reported on intravenous pyelography.

The seven strains described here exhibited six different sensitivity patterns when tested against a standard set of six urinary antimicrobial agents.

Table II. Antibiotic susceptibility of cysteine-requiring isolates of *Klebsiella* tested by the method of Bell (1975)

Organism*	Susceptibility to					
	Sulphonamide	Trimethoprim	Ampicillin	Nalidixic acid	Nitrofurantoin	Gentamicin
<i>K. aerogenes</i>	R	R	R	R	R	R
<i>K. aerogenes</i>	R	R	R	R	R	S
<i>K. aerogenes</i>	S	S	R	S	R	S
<i>K. aerogenes</i>	R	R	R	R	R	S
<i>K. aerogenes</i>	S	S	R	S	S	S
<i>K. aerogenes</i>	S	R	R	S	S	S
<i>K. ozaenae</i>	R	S	R	S	S	S

R = resistant; S = sensitive.

* Identification of isolates according to Cowan (1974).

Figure. Growth yield (A) and specific growth rate (B) of three cysteine-requiring klebsiellae (□, △, ▲) and prototrophic control *K. pneumoniae* ATCC 12657 (■) in Davis and Mingoli medium supplemented with increasing concentrations of L-cysteine.

Several of the auxotrophs appeared to be more resistant to antibiotics than other klebsiellae isolated from the urinary tract over the same period and this may reflect repeated exposure to antimicrobial agents in chronically infected patients. A number of problems arise in testing the sensitivity

of auxotrophic strains *in vitro*. Not all media will fully support the growth of these strains and the presence of cysteine may affect the action of some antibiotics. Nonetheless antibiotic therapy based on the results of the *in-vitro* sensitivity tests appeared to be successful in most cases described

here. One exception was patient 5 who responded clinically and bacteriologically to co-trimoxazole therapy, although the organism appeared to be resistant to this combination *in vitro*.

Cysteine-requiring strains represented about 1.2% of all isolates of *Klebsiella* over the period in which they were identified. This figure is similar to that given for the isolation of cysteine-requiring *E. coli* (1.5% of isolates of this species) in the study of Borderon and Horodniceanu (1978) and indicates that factors responsible for the selection of cysteine auxotrophs in the urinary tract are not restricted to a single genus. This point is further emphasised by the finding that the defect in sulphur metabolism in the *Klebsiella* strains is similar to that defined previously in naturally occurring cysteine-requiring *E. coli* (McIver and Tapsall, 1987) in that the auxotrophic *E. coli* and *Klebsiella* both lack the ability to reduce sulphate to sulphite. This defect imposes a requirement for an exogenous source of suitable sulphur-containing compounds; cysteine and its oxidation product cystine, if present, provide "end product rescue" for the auxotrophs. These compounds have been shown to be present in the urine of fasting patients at concentrations of 2–4 mg/L (16.5–33.0 μ M) and 10–15 mg/L (41.6–62.4 μ M) respectively (Brigham *et al.*, 1960).

The relative lack of reports of cysteine-requiring klebsiellae in comparison to auxotrophic *E. coli* may arise from the much higher rate of isolation of *E. coli* from the urinary tract. Moreover, these

strains may be often misidentified, because failure of some routinely used media to support growth fully will cause difficulties in isolation, identification and sensitivity testing. These problems are partly corrected when exogenous cysteine is available, although Gillespie (1952) noted that additional unidentified factors aid the growth of cysteine-requiring *E. coli*.

We demonstrated that the auxotrophs and the control strain yielded similar growth in the presence of cysteine concentrations of ≥ 20 mg/L. However, the specific growth rate was lower for auxotrophic strains and only approached that of the prototrophic control in the presence of cysteine at the relatively high concentration of 300 mg/L. When the specific growth rates of prototrophic and auxotrophic *E. coli* were compared in earlier experiments (McIver and Tapsall, 1987), no such differences were observed. A further difference between the two genera was observed when toxic effects of higher concentrations of cysteine on *E. coli* and *Klebsiella* were compared. Toxic effects on *E. coli* occurred when the amino acid was present in concentrations of ≥ 250 mg/L (1.59 mM) (McIver and Tapsall, 1987). In contrast, only slight reductions in growth yield and growth rate were observed with either prototrophic or auxotrophic klebsiellae in concentrations of cysteine up to 1000 mg/L.

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REFERENCES

- Bell S M 1975 The CDS disc method of antibiotic sensitivity testing (calibrated dichotomous sensitivity test). *Pathology* 7 Suppl: 1–48.
- Bevis T D 1968 A modified electrolyte deficient culture medium. *Journal of Medical Laboratory Technology* 25: 38–41.
- Borderon E, Horodniceanu T 1978 Metabolically deficient dwarf-colony mutants of *Escherichia coli*: deficiency and resistance to antibiotics of strains isolated from urine culture. *Journal of Clinical Microbiology* 8: 629–634.
- Brigham M P, Stein W H, Moore S 1960 The concentrations of cysteine and cystine in human blood plasma. *Journal of Clinical Investigation* 39: 1633–1638.
- Cowan S T 1974 Cowan and Steel's Manual for the identification of medical bacteria, 2nd ed. Cambridge University Press, Cambridge, pp 103–110.
- Davis B D, Mingioli E S 1950 Mutants of *Escherichia coli* requiring methionine or vitamin B₁₂. *Journal of Bacteriology* 60: 17–28.
- Gillespie W A 1952 Biochemical mutants of coliform bacilli in infections of the urinary tract. *Journal of Pathology and Bacteriology* 64: 551–557.
- Jones-Mortimer M C 1968 Positive control of sulphate reduction in *Escherichia coli*. Isolation, characterization and mapping of cysteine-less mutants of *E. coli* K12. *Biochemical Journal* 110: 589–595.
- Leigh D A, Williams J D 1964 Method for the detection of significant bacteriuria in large groups of patients. *Journal of Clinical Pathology* 17: 498–503.
- Mackey J P, Sandys G H 1966 Diagnosis of urinary infections. *British Medical Journal* 1: 1173.
- McIver C J, Tapsall J W 1987 Cysteine requirements of naturally occurring cysteine auxotrophs of *Escherichia coli*. *Pathology* 19: 361–363.
- Miles A A, Misra S S, Irwin E D 1938 The estimation of the bactericidal power of the blood. *Journal of Hygiene* 38: 732–749.
- Nagy Z, Kari C, Hernádi F 1969 Growth of *Escherichia coli* cells in the presence of cysteine on sulphate-deficient media. *Archiv für Mikrobiologie* 65: 391–400.
- Pasternak C A 1962 Sulphate activation and its control in *Escherichia coli* and *Bacillus subtilis*. *Biochemical Journal* 85: 44–49.
- Tapsall J W, McIver C J 1986 Septicaemia caused by cysteine-requiring isolates of *Escherichia coli*. *Journal of Medical Microbiology* 22: 379–382.
- Tapsall J W, Wilson E, Harper J 1974 Thymine dependent strains of *Escherichia coli* selected by trimethoprim-sulphamethoxazole therapy. *Pathology* 6: 161–167.