Assessment of Conventional and Commercial Methods for Identification of Clinical Isolates of Cysteine-Requiring Strains of *Escherichia coli* and *Klebsiella* Species

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Cysteine-requiring strains of the family *Enterobacteriaceae* that are auxotrophic for this amino acid because of defects in the sulfur assimilatory pathway account for about 1.5% of urinary tract isolates of *Escherichia coli* and *Klebsiella* species. Forty *Escherichia* and eight *Klebsiella* cysteine-requiring strains were used to test the ease with which various test systems identified clinical isolates of cysteine auxotrophs. In a preliminary experiment, the growth yield of 10 cysteine-requiring *E. coli* in 10 solutions of commercially available peptones was in each instance less than that of prototrophic control and showed that these sources of nutrients were suboptimal for these strains. A significant proportion of the cysteine-requiring strains were not adequately identified by growth-dependent tests which used various peptones as a nutrient source. Problems were encountered with all test systems examined, which were as follows: conventional methods; the API 20E, Microbact, and Vitek systems; and two rapid methods for the identification of *E. coli*, the Rapidec coli and the β -D-glucuronidase tests. The performance of the test systems was only partly improved when inocula were derived from appropriately supplemented media. However, the problems of the growth-dependent tests were resolved when a cysteine-supplemented suspension was used to inoculate each test system.

Strains of the family *Enterobacteriaceae* auxotrophic for a variety of nutrients are not infrequently isolated from clinical specimens. Such organisms often grow poorly on isolation media and form so-called dwarf colonies (2). Strains of *Escherichia coli* which produce dwarf colonies represent about 2% of all isolates of this species from urinary tract infections (2) and are more frequently recovered from elderly patients with long-standing urinary tract abnormalities (2, 7) or from patients on long-term co-trimoxazole therapy (20). The isolates from the former group of patients, termed cysteine requiring by Gillespie (7), have been shown to lack the ability to assimilate sulfate. Strains of *E. coli* which show this defect have been isolated from cultures of blood and wound abscesses as well as from urine (19), and isolates of *Klebsiella* species with the same defect have also been shown to be responsible for urinary tract infections (14).

A functional sulfur assimilatory pathway results in the formation of cysteine, and end product rescue with this amino acid or its oxidation product, cystine, will rectify the defect caused by the lack of sulfate assimilation in cysteine auxotrophs. Laboratory media used routinely in clinical laboratories commonly incorporate peptones in their constituents. These peptones are not fully characterized with regard to their amino acid content or concentration. Cysteine is normally denatured during the production of peptones, so that cystine-supplemented media have been developed to facilitate the isolation of cysteine auxotrophs (1, 12). The amounts of cysteine which allow the maximal growth yields of auxotrophs but without toxic effects on the growth of auxotrophic and prototrophic strains of *E. coli* have been determined in chemically defined media (13). However, media used for identification of members of the *Enterobacteriaceae* are routinely supplemented with cysteine or cystine.

We have noted some problems with growth-dependent

tests in conventional and commercial identification systems which were resolved following the addition of cysteine to identification media (14, 19), and these problems are further explored in this investigation. We report here the results of experiments in which we tested and compared the abilities of various commercially available peptones to support the growth of cysteine-requiring *E. coli*. Further, the performance of conventional identification schemes which utilize media containing some of these peptones was assessed with a larger number of cysteine-auxotrophic strains of *E. coli* and *Klebsiella* species. Additionally, the problems previously encountered when some commercial systems failed to fully identify these strains were examined more extensively by testing the facility with which the API 20E, the Microbact system, and the Vitek computer-assisted identification procedure identified cysteine-auxotrophic *E. coli* and *Klebsiella* species. Two rapid methods used to identify *E. coli*, namely, the Rapidec coli (API) and the β -D-glucuronidase test (8), were also examined for their ability to identify these auxotrophs. On the basis of these findings, we discuss adaptations of laboratory procedures which enhance the identification to the species level of these strains.

MATERIALS AND METHODS

Strains. A total of 48 strains auxotrophic for cysteine were used in these experiments. All were isolated from clinical samples, predominantly from infected urine, but isolates from blood cultures and wound abscesses were also included. Twelve strains (five *E. coli* and seven *Klebsiella* species) have been previously described (13, 14, 19). All strains were originally isolated on MacConkey agar (Oxoid Ltd., Basingstoke, England, and Difco Laboratories, Detroit, Mich.), where they grew as weak lactose-fermenting dwarf colonies. Their requirement for cysteine was demonstrated by an auxanographic technique in which Sensitest agar (Oxoid) and paper disks impregnated with a solution containing 0.63 mM (100 mg/liter) L-cysteine HCl were used

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(Sigma Chemical Co., St. Louis, Mo.) (19). Protrophic strains *E. coli* NCTC 10418 and *Klebsiella pneumoniae* ATCC 12657 were used as controls in these experiments.

Growth of cysteine-requiring *E. coli* strains in various peptone waters. Ten cysteine-requiring strains which were identified by conventional methods (3) as *E. coli* were examined for their ability to grow in aqueous solutions of the following peptones: bacteriological peptone (L007/71036 P501; Evans Medical, Liverpool, England), bacteriological peptone (L37; Oxoid), Bacto-Peptone (0118-01-8; Difco), casein hydrolysate (L41; Oxoid), neopeptone (0119-01; Difco), peptone-P (L49; Oxoid), Proteose Peptone (0120-01; Difco), Proteose Peptone no. 2 (0121-01; Difco), Proteose Peptone no. 3 (0122-01; Difco), and special peptone (L72; Oxoid). Two different batches of each peptone were tested.

The growth response, as measured by growth yield after 24 h of incubation, of the 10 auxotrophs was compared with that of a protrophic control, *E. coli* NCTC 10418. Peptone water solutions were prepared by dissolving 10 g of each peptone and 5 g of sodium chloride in separate 1-liter volumes of water. In addition, a solution of Bacto-Peptone (Difco) was prepared with L-cysteine HCl (0.63 mM, final concentration). Each solution was adjusted to pH 7.4. These solutions were sterilized by autoclaving and dispensed in 3-ml volumes into sterile glass tubes (13 by 100 mm). A suspension of each auxotroph was made by taking three dwarf colonies from MacConkey agar plate and suspending them in 2.5 ml of physiological saline. A volume of 0.025 ml of this suspension was used as the inoculum. The inoculum size, determined by means of surface viable counts (15), was on the order of 10^4 CFU for each strain and control. The growth yield was determined by measuring the optical density of the bacterial suspension in each tube at a wavelength of 640 nm by using a spectrophotometer (model Spectronic 20; Milton Roy, Rochester, N.Y.) after 24 h of incubation in air at 37°C. The growth yield, as measured by optical density, was previously related to surface viable counts (13). Each strain was assessed in duplicate, and the experiments were repeated once. The level of probability of a significant difference between the growth yields of the auxotrophs obtained in Bacto-Peptone (Difco) with and without cysteine was determined by using Student's *t* test for unpaired observations.

Identification methods. All 48 strains were used in this series of investigations. The following tests were done with the media and methods described by Cowan (5): utilization of 1% solutions of glucose, lactose, salicin, mannitol, and maltose (incorporating Difco Bacto-Peptone); citrate utilization in Simmons citrate agar (Oxoid) (18); lysine decarboxylase test of Moeller (16); methyl red and Voges-Proskauer (incorporating Difco Bacto-Peptone); nitrate reduction (incorporating Oxoid nutrient broth no. 2); *o*-nitrophenyl- β -D-galactopyranoside (incorporating Difco Bacto-Peptone) of Lowe (11); Hugh and Leifson oxidation-fermentation test (9) (incorporating Evans bacteriological peptone); phenylalanine deamination (Difco); and Christensen's test for urea hydrolysis (4) with Oxoid agar base (incorporating Oxoid bacteriological peptone). Kligler iron agar (Oxoid) was used to detect differential utilization of dextrose and lactose and hydrogen sulfide production. Motility was detected in semi-solid media as described by Edwards and Ewing (6), with a supplement of 1% tryptone (Oxoid) to test for indole production.

The tests were inoculated from suspensions prepared in saline alone (as above) and saline with a supplement of L-cysteine HCl (0.63 mM). All tests were incubated in air at

37°C, and results were recorded after 24 and 48 h of incubation. The identification was determined by using the scheme for members of the *Enterobacteriaceae* in *Bergey's Manual of Systematic Bacteriology*, volume 1 (3).

API 20E. The API 20E (API System, Montalieu Vercieu, France) is a dehydrated test strip to which an inoculum of a bacterium being tested is added. The kit was used in accordance with the accompanying instruction manual (version C). Inocula were derived from suspensions of organisms in 5 ml of sterile water alone and sterile water supplemented with 0.63 mM L-cysteine HCl. Suspensions were prepared by using three dwarf colonies from growth on MacConkey agar. For inocula prepared from CLED agar (1, 12), one normal-sized colony was used and it provided an inoculum of similar cell density. The test reactions were initially recorded after 24 h of incubation and then after 48 h for detection of weak reactions. The profile numbers thus generated were used to identify these strains by using the Analytical Profile Index catalog for the *Enterobacteriaceae* and other gram-negative bacteria.

Microbact. The Microbact 12 E system (Disposable Products, Ridleyton, South Australia, Australia) comprises dehydrated substrates to which an inoculum of a bacterium under test is added. The kit was used in accordance with the instructions of the manufacturer. Organisms were grown overnight on both MacConkey (Oxoid) and CLED (Oxoid) agar plates. Each test strip was inoculated with a suspension prepared from a normal-sized colony on CLED agar in 1.5 ml of saline. As recommended, a single colony from a MacConkey plate was inoculated into 3 ml of peptone water and incubated for 4 h. After this time, the organism was suspended in saline and a test strip was inoculated. In this study, two inocula were prepared, one from growth in peptone water with Bacto-Peptone (Difco) alone and the other from Bacto-Peptone supplemented with L-cysteine HCl (0.63 mM). The reaction profile obtained after 24 h of incubation was recorded, and the organisms were identified by means of the Microbact system profile register for members of the *Enterobacteriaceae*.

Vitek. The Vitek method uses a system of computer analysis of growth response and reactions to a number of substrates incorporated in a test card to identify a variety of microorganisms. For these experiments, the auxotrophic strains were grown on MacConkey (Oxoid) and CLED (Oxoid) agars. Test strains harvested from each agar were suspended in 0.45% sterile saline so as to produce an inoculum equivalent in turbidity to a McFarland no. 1 turbidity standard. In addition, auxotrophs growing on MacConkey agar were suspended in a separate saline suspension supplemented with L-cysteine HCl (0.63 mM). The Vitek systems Gram Negative Identification (GNI) card was used according to the instructions of the manufacturer.

Rapidec coli. The Rapidec coli (API System) is a rapid method for the presumptive identification of *E. coli*. The test was applied according to the instructions included in each kit (version A) to identify fresh cultures of cysteine-requiring strains of *E. coli* grown on MacConkey and CLED agars. The reactions were recorded after 2 h of incubation and interpreted according to the specifications of the manufacturer.

β -D-Glucuronidase. The method of Hansen and Yourasowksy (8) was applied to detect preformed glucuronidase activity in the auxotrophic strains of *E. coli*. Tests were performed by adding to a 0.5-ml suspension of each strain (McFarland standard no. 4), a paper disk containing 0.05 ml of a 1% solution of *p*-nitrophenol- β -D-glucuronide (Sigma)

FIG. 1. Growth yield of 10 cysteine-auxotrophic *E. coli* compared with the growth yield of prototrophic *E. coli* NCTC 10418 in 1% peptone water solutions. The growth response of the auxotrophs is shown as the mean of the absorbance of duplicate examinations repeated once, plus or minus one standard deviation (s.d.). Sources of peptone: 1, bacteriological peptone (Evans); 2, bacteriological peptone (Oxoid); 3, Bacto-Peptone (Difco); 4, Bacto-Peptone (Difco) supplemented with 0.63 mM L-cysteine HCl; 5, casein hydrolysate (Oxoid); 6, peptone-P (Oxoid); 7, neopeptone (Oxoid); 8, Proteose Peptone (Difco); 9, Proteose Peptone no. 2 (Difco); 10, Proteose Peptone no. 3 (Difco); 11, special peptone (Oxoid).

prepared in 0.06 M Sorensen phosphate buffer (pH 8). Cysteine-requiring strains of *E. coli* were tested from subcultures grown overnight on cysteine-supplemented brain heart infusion agar (Difco) (0.63 mM L-cysteine HCl) and MacConkey and CLED agars. Glucuronidase activity was indicated by the presence of a yellow color in the supernatant after 2 h of incubation and was reexamined after 24 h of incubation at 37°C.

RESULTS

Growth in peptone water. The growth yield of 10 cysteine-requiring *E. coli* and a prototrophic *E. coli* grown in 11 different commercial peptone water solutions was recorded after 24 h of incubation (Fig. 1). The means and standard deviations of the growth yields of the 10 auxotrophs in each solution are shown. Overall, the means of the growth yields of the auxotrophs in each solution were lower than the growth yield obtained with the prototroph. Bacteriological peptone (Oxoid) and casein hydrolysate (Oxoid) gave the lowest growth yields by the auxotrophs. The highest growth yield for both the auxotrophs and the prototrophic strains was obtained in Proteose Peptone no. 2 (Difco). The growth yields obtained by the auxotrophs in Bacto-Peptone (Difco) were not significantly different from the yields obtained in the same medium with added cysteine ($0.375 < P < 0.4$). The growth yield of the prototrophic control in Bacto-Peptone was reduced in the presence of added cysteine.

Identification of cysteine auxotrophs. The results of the final identifications of the cysteine-requiring strains by using conventional reactions and the API 20E, Microbact, and Vitek systems with unsupplemented inocula derived from MacConkey and CLED agars and cysteine-supplemented inocula are shown in Table 1. There was general agreement between the various systems that 40 of the strains should be regarded as *E. coli* and 8 as *Klebsiella* species.

There was a high concordance of results for the 40 strains identified as *E. coli* when conventional reactions and the API 20E profiles were compared. With the API 20E system,

differences were observed with most strains in profile numbers obtained with cysteine-supplemented and unsupplemented inocula after 24 h of incubation. The profile numbers obtained with unsupplemented inocula changed after 48 h of incubation and were identical with those obtained with cysteine-supplemented reactions after 24 h. The differences were due to the lower growth rate in one or more of the carbohydrate fermentation tests. Despite this difference in profile numbers, all isolates were ultimately identified as *E. coli*, irrespective of the inocula used.

There was a high correlation of results obtained with the Microbact system and the Vitek method with conventional and API 20E test results, but only when cysteine supplementation was used. The Microbact system identified all these 40 strains as *E. coli*, irrespective of the derivation of the inoculum, but a greater degree of precision was obtained with cysteine supplementation. This was evident when 19 of these 40 isolates were identified as "*E. coli* inactive" by using an unsupplemented inoculum. Use of CLED agar to derive the inoculum for this system reduced the number of inactive strains substantially. With the Vitek system, however, only the use of a cysteine-supplemented inocula gave a satisfactory result. By using unsupplemented inocula from MacConkey agar, 11 of the 40 strains were not identified as belonging to the genus *Escherichia*. Similarly, 8 of the 40 strains were misidentified with inocula derived from CLED agar. With use of the cysteine-supplemented inoculum, the average time for identification by the Vitek system was reduced from 13 h with the standard preparation to 5 h with the cysteine-supplemented variation. With added cysteine, all strains were identified as belonging to the *Escherichia* genus, including one strain categorized as belonging to the A-D group.

There was a greater degree of discrepancy among the test systems when the identifications of the more biochemically active strains of *Klebsiella* species were examined. A satisfactory correlation between the results obtained with the conventional identification method and the API 20E and the

TABLE 1. Final identification of cysteine-requiring strains of *E. coli* ($n = 40$) and *Klebsiella* species ($n = 8$) by conventional and commercial methods^a

Organism or group	No. of strains identified by:										
	Conventional methods ^b		API 20E			Microbact			Vitek		
	A	B	C	D	E	F	G	H	I	J	K
<i>Escherichia coli</i>	38	38	38	38	38	21	38	35	29	31	39
<i>Escherichia coli</i> (inactive)	2	2				19	2	5			
<i>Escherichia</i> group A-D			2	2	2				0	1	1
Other	0	0	0	0	0	0	0	0	9	8	0
Unidentifiable	0	0	0	0	0	0	0	0	2	0	0
<i>Klebsiella pneumoniae</i>	7	7	1	2	7	1	7	2	7	7	7
<i>Klebsiella ozaenae</i>	1	1						1	1	1	1
<i>Klebsiella</i> species ^c			6	4	1						
<i>Klebsiella-Enterobacter-Serratia</i> group ^d						7	1	6			
Unidentifiable	0	0	1	2	0	0	0	0	0	0	0

^a Inocula were as follows: A, unsupplemented, with growth obtained on MacConkey agar; B, supplemented with cysteine, with growth obtained on MacConkey agar; C, unsupplemented, with growth from MacConkey agar (tests were incubated up to 48 h to enhance positive reactions); D, unsupplemented, with growth from CLED agar (tests were incubated up to 48 h to enhance positive reactions); E, supplemented with cysteine, with growth obtained on MacConkey agar; F, with growth obtained on MacConkey agar and grown in peptone water before inoculation; G, with growth obtained on MacConkey agar and grown in cysteine-supplemented peptone water before inoculation; H, with growth obtained on CLED agar; I, unsupplemented, with growth obtained on MacConkey agar; J, unsupplemented, with growth obtained on CLED agar; K, supplemented with cysteine, with growth obtained on MacConkey agar.

^b Strains were identified in conventional media by using the criteria of *Bergey's Manual of Systematic Bacteriology*, volume 1 (3).

^c Strains were not identified to species level by method.

^d The probability of an accurate identification is low and requires further testing to increase specificity (Microbact).

Microbact methods was obtained only when cysteine supplementation of inocula was used. The auxotrophs failed to grow on conventional media used to test urease production and citrate utilization unless cysteine supplementation was provided. While failure of the strains to grow on the urease and citrate utilization test media did not affect the final identification, the reactions would have enhanced the probability of accuracy in the identification of the auxotrophic *Klebsiella* species.

Cysteine supplementation was also necessary for the identification of the *Klebsiella* strains by the API 20E system. In particular, the carbohydrate fermentation tests and the Voges-Proskauer reaction were promoted by cysteine supplementation. The Microbact system placed most of the *Klebsiella* strains in the *Enterobacter-Klebsiella-Serratia* group, which required further testing, when inocula from CLED media were used. The choice of peptone water to increase the inoculum size before inoculating the Microbact test was also demonstrated to influence the identification. The *o*-nitrophenyl- β -D-galactopyranoside, urease, Voges-Proskauer, and carbohydrate fermentation reactions were either negative or slow to react in tests of inocula derived from peptone water without cysteine. The Vitek system identified all strains irrespective of the source of the inoculum. There was no significant difference in the identification time when supplemented and unsupplemented inocula were used. Conversely, the use of cysteine supplementation did not hinder the identification of these strains nor did it hinder identification of the prototrophic control.

Rapid methods. Of the 38 strains of *E. coli* which were identified as *E. coli* by the methods described above, the Rapidec coli (API) identified 29 with inocula obtained from CLED agar and 27 with inocula prepared from MacConkey agar. Two strains identified as *E. coli* inactive by conventional methods were identified as probable *E. coli*.

Glucuronidase activity was detected in 33 of the 40 auxotrophic *E. coli* tested. When grown on cysteine-supplemented brain heart infusion agar or on CLED agar, the same 33 strains gave a positive glucuronidase test. Thirty-two of

these strains gave a positive result when the test strains were grown on MacConkey agar.

DISCUSSION

We have previously described some difficulties encountered with the identification of strains of the *Enterobacteriaceae* auxotrophic for cysteine in conventional and commercial systems (14, 19). These observations were confirmed and extended in this study with a larger number of isolates and commercial identification systems when a significant proportion of cysteine-requiring strains of *E. coli* and *Klebsiella* species were not adequately identified. Although both conventional tests and the API 20E ultimately identified the 40 strains of auxotrophic *E. coli*, it was necessary to use extended incubation times to overcome problems related to slow growth of the strains in a deficient basal medium. Difficulties were also encountered with the other commercial systems and rapid identification methods for auxotrophic *E. coli*. With the more biochemically active *Klebsiella* species, the API 20E and the Microbact systems showed a decreased capacity to correctly identify these strains. In contrast, the Vitek system performed well in this aspect of the study, but for optimal identification of *E. coli* with this system, cysteine supplementation of inocula was necessary.

These problems can be attributed to the poor growth of the auxotrophs in growth-dependent tests, this being a consequence of the lack of appropriate nutrients for these strains in peptones, which are a basic component of the media in the test systems. The growth yields of the auxotrophic strains were considerably less than that of the prototrophic control in the peptones tested. High yields of the auxotrophic strains were obtained in Bacto-Peptone (Difco), Evans bacteriological peptone, Proteose Peptone no. 2 (Difco), and special peptone (Oxoid). The use of Bacto-Peptone (Difco) and Evans bacteriological peptone in conventional identification media enhanced the reactions of the auxotrophs without cysteine supplementation. The lowest growth yield was obtained by using Oxoid bacteriological peptone. This pep-

tone is a constituent of a number of laboratory media supplied by this manufacturer and requires cysteine supplementation for adequate growth of these strains.

Interestingly, the addition of cysteine to Bacto-Peptone (Difco) not only failed to increase the growth yields of auxotrophs in this medium but actually decreased the yield of the prototrophic control when grown under identical conditions. This phenomenon may be explained by postulating that this peptone is rich in cysteine and that additional supplementation may have resulted in cysteine toxicity. Higher levels of this amino acid are known to decrease growth yields of both auxotrophic (13) and prototrophic (17) *E. coli*, but these effects may in turn be moderated by the presence of branched chain amino acids. Further studies of this phenomenon were not pursued here because of the difficulties inherent in investigations in which complex but relatively undefined peptone water solutions are used. Rather, the information gathered from these experiments and the data on the deficient growth-promoting potential of the peptones studied suggested other means of overcoming these problems.

One such approach was the supplementation of suspensions of the organism being tested with 0.63 mM L-cysteine HCl. These suspensions, used to inoculate the various test systems, contained amounts of cysteine shown previously to increase growth yields under defined conditions. The success of this approach was shown in the comparative studies reported here. For all test systems examined, the identification of strains either became possible or else was facilitated in the presence of cysteine supplementation. Strains of *E. coli* and *Klebsiella* species were identified by all systems with cysteine supplementation with a greater degree of precision, and the time to give a positive result was greatly reduced. For example, the profile number recorded with the API 20E system was obtained in 24 rather than 48 h and the time for identification by the Vitek system was reduced from an average of 13 to 5 h. It is also emphasized that no adverse effects on growth in the test systems were observed with the level of cysteine used. We also investigated the possibility of using inocula from cysteine- or cystine-enriched isolation media as a means of overcoming the problems of growth in cysteine-deficient identification media. In these experiments, subcultures of the auxotrophs were made onto CLED agar, and for the examination of the glucuronidase test, subcultures were made onto a cysteine-enriched nutrient medium. Inocula derived from these media only marginally improved the performance of the various test systems. It would seem that residual cysteine-cystine levels in the strains subcultured from this type of medium were insufficient to maintain growth on nutritionally deficient identification media.

It is difficult for a number of reasons to estimate the extent of the problems posed by organisms of this type to a diagnostic laboratory. One report indicated that about 1.5% of all *E. coli* isolated from the urinary tract are cysteine requiring (2). Our experience over the past 4 years would indicate a similar rate of isolation of *E. coli* of this type from this source (unpublished data). Additionally, strains of cysteine-requiring *Klebsiella* species represent about 1.2% of all isolates of *Klebsiella* species of urinary tract origin in our laboratory (14). The methods whereby these strains may be recognized as auxotrophs by simple procedures have been described previously (14, 19, 20). The means by which laboratory procedures can be adapted to fully identify them are also simple and should be used when auxotrophy is suggested on the basis of unusual colonial morphology or

when inadequate growth occurs in identification or susceptibility testing systems. By these means, it is hoped that a more widespread recognition of these strains will occur and that their role in disease processes will be elucidated.

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