Development of Specific Tests for Rapid Detection of *Escherichia coli* and all Species of *Proteus* in Urine

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Proteus mirabilis was distinguishable from *Escherichia coli* and from several other species that may be associated with urinary tract infections when grown in a nutrient medium supplemented with 0.1 M L-methionine by the formation of large amounts of dimethyl disulfide and methyl mercaptan, which were detected by head-space gas-liquid chromatography (HS-GLC). *E. coli* could be detected by the same HS-GLC technique by ethanol production from methionine peptone water enriched with 1% either lactose or arabinose but not by any product from 10 amino acids tested. Ethanol from lactose was detected early in the exponential phase of growth. Significant numbers, 10^5 or more per ml, of *E. coli* in urine could be detected in about 5 h by ethanol production from an unshaken culture of urine in lactose methionine peptone water buffered at pH 7.2 (urine test medium); only a trace of dimethyl disulfide was produced. Significant numbers of *P. mirabilis* in urine could be detected in 4 h by dimethyl disulfide production and in 5 h by methyl mercaptan production from a shaken culture of urine in urine test medium; no ethanol was produced. Incubation of urine specimens in the test medium followed by examination by HS-GLC is proposed as a rapid method of detecting whether or not the urine contains significant numbers of *E. coli* or a species of *Proteus*.

It has been shown (6) that dimethyl disulfide is formed in large amounts early in the growth of all six species of *Proteus* (*Proteus mirabilis*, *P. vulgaris*, *P. morganii*, *P. rettgeri*, *P. inconstans* A, and *P. inconstans* B) in media supplemented with 0.1 M L-methionine. Dimethyl disulfide can be detected readily by head-space (HS) gas-liquid chromatography (GLC; HS-GLC). This suggests that the detection of dimethyl disulfide by HS-GLC from cultures in methionine media could be used to give an early indication of the presence of *Proteus* species.

An important application of a rapid method for detection of *Proteus* spp. would be in diagnosing urinary tract infections. However, before the detection of dimethyl disulfide from methionine can be proposed as a method for indicating *Proteus* spp. in urine, it must be shown that other organisms found in urine do not metabolize methionine as these species do. Also, *Proteus* spp. are less common than *Escherichia coli* in urine, and it would be highly desirable to be able to detect and differentiate

E. coli by a product not formed by *Proteus* spp., preferably in the same HS-GLC test. The test would need to be sufficiently quantitative to distinguish between counts of 3×10^3 bacteria per ml of urine, the usual upper limit of contamination, and 10^5 bacteria per ml of urine, representing "significant bacteriuria" (3, 7).

MATERIALS AND METHODS

Bacterial cultures. The identities of strains of *E. coli*, *Klebsiella aerogenes* (*Klebsiella pneumoniae*), *K. oxytoca* (*K. pneumoniae*), *K. ozaenae*, *Citrobacter freundii*, *Enterobacter cloacae*, *Serratia marcescens*, *P. mirabilis*, *Staphylococcus aureus*, *S. epidermidis*, and *Streptococcus faecalis* isolated from clinical specimens or obtained from culture collections were confirmed by standard tests (4).

Culture media. The basal defined medium containing lactate, amino acids, salts, and vitamins (LAS), either plain or supplemented with amino acids, was prepared as described previously (6).

Yeast extract broth (YEB) was prepared from nutrient broth no. 2 (Oxoid) and 0.3% (wt/vol) yeast extract powder (Oxoid) and supplemented with 0.1 M L-methionine by dissolving the amino acid in YEB and sterilizing by sintered-glass filtration.

Peptone water (PW) was prepared from bacteriological peptone (Oxoid) by a standard method (5) and supplemented with 0.1 M L-methionine and 1%

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(wt/vol) of either lactose or arabinose, by dissolving the supplement in the medium and sterilizing by filtration.

MacConkey agar (Oxoid) was prepared as directed by the manufacturer.

Ingredients of urine test medium. The buffer was 0.2 M potassium dihydrogen phosphate-disodium hydrogen phosphate at pH 7.2 sterilized by autoclaving at 121°C for 20 min. The specific test substrates were prepared by dissolving 0.4 M L-methionine and 4% (wt/vol) lactose in buffer at 60°C and sterilizing while hot by filtration. Double-strength PW, 2% (wt/vol), was prepared from bacteriological peptone (Oxoid) and sterilized by autoclaving at 121°C for 20 min. The complete medium was prepared by mixing equal volumes of specific test substrates and double-strength PW and was used with an equal volume of urine in the test. The final concentrations of ingredients were: L-methionine, 0.1 M; lactose, 1%; phosphate buffer, 0.05 M; peptone, 0.5%; and urine, 50%.

Incubation conditions. Unless otherwise specified, all media were incubated at 37°C. LAS and YEB media were dispensed with sterile precautions in 20-ml samples in sterile 125-ml Erlenmeyer flasks stoppered with cotton-wool plugs and incubated either still or shaking in a rotary shaker at 160 rpm. The urine test medium was dispensed with sterile precautions in 5-ml amounts in sterile Universal bottles, and 5 ml of urine was added. PW media were dispensed in 10-ml amounts in Universal bottles. Bottles for shaken cultures were stoppered with cotton-wool plugs, and those for still cultures were stoppered with screw caps.

Measurement of bacterial growth, preparation of HS samples for GLC, and the operation of the gas chromatograph. These were the same as described previously (6).

Estimation of products. Three products not previously identified, ethanol, ethyl methyl ketone, and n-propanol, were encountered and identified by

their mass spectra and GLC retention times. Amounts of products were calculated in the same way as previously described (6).

RESULTS

HS products formed from L-methionine by urinary tract organisms other than *Proteus* spp. LAS medium supplemented with 0.1 M L-methionine was inoculated with approximately 0.01 ml of liquid cultures of *E. coli* (six strains), *C. freundii*, *E. cloacae*, *S. marcescens*, *K. aerogenes*, *P. aeruginosa*, *S. aureus*, *S. epidermidis*, *S. faecalis*, or *P. mirabilis* and incubated while shaking for 18 h. The gram-positive cocci failed to grow. The results of the HS analyses of the cultures of the gram-negative bacilli for dimethyl disulfide and methyl mercaptan are shown in Table 1. All of the organisms formed dimethyl disulfide, but *P. mirabilis* formed the largest amount, 50 times more than any strain of *E. coli* and at least four times the yield of any other culture tested. Methyl mercaptan was detected only from *P. mirabilis*, apart from a trace from *C. freundii*. It can be concluded that all *Proteus* spp. would be more active in producing sulfur products than the other bacteria tested, because earlier work with a number of strains of all species of *Proteus* firmly established that there is very little variation within this genus in the formation of sulfur products from methionine (6).

YEB medium supplemented with 0.1 M L-methionine was inoculated in the same way with the same organisms and incubated shaking for 18 h. All of the cultures grew. The

TABLE 1. Sulfur products formed from 18-h shaken cultures in media supplemented with 0.1 M L-methionine by various bacteria found in urine

Species	Peak area (cm ²)			
	LAS medium		YEB medium	
	Dimethyl disulfide	Methyl mercaptan	Dimethyl disulfide	Methyl mercaptan
<i>P. mirabilis</i>	690	452	443	104
<i>E. coli</i>				
Strain 1	11	0	56	0
Strain 2	18	0	128	0
Strain 3	11	0	107	0
Strain 4	16	0	172	0
Strain 5	16	0	206	0
Strain 6	5	0	83	0
<i>C. freundii</i>	177	1	178	2
<i>E. cloacae</i>	8	0	7	0
<i>S. marcescens</i>	131	0	146	0
<i>K. aerogenes</i>	175	0	32	0
<i>P. aeruginosa</i>	73	0	25	0
<i>S. aureus</i>	— ^a	—	0	0
<i>S. epidermidis</i>	—	—	0	0
<i>S. faecalis</i>	—	—	4	0

^a —, Cultures failed to grow.

results of analyses are shown in Table 1. No dimethyl disulfide was formed by *S. aureus* or *S. epidermidis*, and only a trace was formed by *S. faecalis*. The relative amounts of dimethyl disulfide formed by the other organisms were different from the relative amounts from LAS medium, but *P. mirabilis* still formed the largest amount and was the only culture to yield methyl mercaptan, apart from a trace from *C. freundii*. The optical densities of cultures in LAS and YEB media were found to be similar, so that the larger yields of dimethyl disulfide from the *E. coli* cultures in YEB were not due to more growth.

It was concluded that, under controlled conditions, *Proteus* spp. would probably be clearly distinguishable from other urinary bacteria by their higher yields of dimethyl disulfide and by their yields of methyl mercaptan.

HS products formed by *E. coli*. A search was made for products formed by *E. coli* that were detectable under the HS-GLC conditions that distinguished *Proteus* spp. from other bacteria commonly associated with urinary tract infections.

LAS medium supplemented with various L-amino acids was inoculated with *E. coli*, incubated shaking for 18 h, and analyzed for HS products. No products were detectable from 0.1 M leucine, phenylalanine, histidine, lysine, ornithine, or asparagine, or from 0.05 M aspartic acid or tryptophan. Moderate amounts of isobutanol were detected from 0.1 M valine, and a small amount of an unidentified compound at retention time 12 min was detected from 0.1 M threonine. Since *Proteus* spp. are known to form isobutanol from valine (6), and the compound from threonine, though not detected before, was present in only a small amount, amino acids did not appear to be useful substrates for the specific detection of *E. coli* by HS analysis of products formed under these cultural conditions.

The usefulness of carbohydrates as substrates was investigated by supplementing methionine PW with either lactose or arabinose, both of which are not fermented by *Proteus* spp. (4), inoculating with 19 strains of lactose-fermenting enterobacteria and one of *S. marcescens*, and incubating, both shaking and still, for 18 h before analysis by HS-GLC. All strains of *E. coli* were consistent in forming ethanol (Table 2) from both sugars in still cultures. Ethanol production was variable and in lower yields from shaken cultures. All strains of *K. aerogenes* and *K. oxytoca* also formed ethanol from both sugars in still and shaken cultures, but the amounts produced differed quantitatively from *E. coli*. The range of yields from

lactose was wider than that for *E. coli*; yields from shaken cultures were similar to those from still cultures. The single strains of *K. ozaenae* and *E. cloacae* were more like *E. coli* in that yields of ethanol from both sugars were similar and were smaller from shaken than from still cultures. *S. marcescens* did not yield ethanol from any culture. Ethanol was not formed by any species in control cultures in methionine PW.

Small amounts of *n*-propanol were formed in still cultures from both sugars by all strains of *E. coli*, two of *K. aerogenes*, by *K. ozaenae*, and by *E. cloacae*.

All species yielded dimethyl disulfide from shaken cultures in methionine PW (Table 3), but the amount was small compared with *P. mirabilis* (Table 1) and was less in media supplemented with either sugar. No methyl mercaptan was detected.

Ethanol production from lactose in still cultures appeared to be a constant property of *E. coli* and a variable property of other lactose-fermenting enterobacteria. It appeared probable that this reaction could be used to detect *E. coli* but that some strains of other enterobacteria would give positive results. Ethanol production from arabinose in still cultures appeared to be a property of all strains of all lactose-fermenting enterobacteria tested. Although ethanol production from arabinose could probably be used to detect *E. coli*, the production of this alcohol from lactose appeared to be of greater value because fewer strains of other enterobacteria would be confused with *E. coli*. Therefore, lactose was selected as the substrate for further investigation.

Still cultures of about 10^6 *E. coli* per ml in lactose PW were examined by HS-GLC, and

TABLE 2. Ethanol production from 18-h cultures in methionine PW supplemented with lactose or arabinose

Species	No. of strains	Mean peak area (cm ²)			
		Lactose methionine PW		Arabinose methionine PW	
		Still	Shaken	Still	Shaken
<i>E. coli</i>	6	94 58-129 ^a	24 12-52	90 58-148	24 13-46
<i>K. aerogenes</i>	8	76 10-206	87 13-265	156 90-232	262 161-516
<i>K. oxytoca</i>	3	82 9-145	24 7-58	160 131-203	139 15-377
<i>K. ozaenae</i>	1	187	41	174	31
<i>E. cloacae</i>	1	406	46	465	35
<i>S. marcescens</i>	1	0	0	0	0

^a Range of peak areas.

TABLE 3. Dimethyl disulfide production from 18-h cultures in methionine PW either plain or supplemented with lactose or arabinose

Species	No. of strains	Mean peak area (cm ²)					
		Methionine PW		Methionine lactose PW		Methionine arabinose PW	
		Still	Shaken	Still	Shaken	Still	Shaken
<i>E. coli</i>	6	0	16	0	4	0	6
<i>K. aerogenes</i>	8	0	27	0	1.8	1.2	19
<i>K. oxytoca</i>	3	0	1.4	0	0	0	0
<i>K. ozaenae</i>	1	0	19	0	12	0	12
<i>E. cloacae</i>	1	0	6.2	0	0	0	1.9
<i>S. marcescens</i>	1	0	26	0	14	0	18

viable counts were determined in a time-course study (Fig. 1). Ethanol and *n*-propanol production was detected 2 to 4 h after inoculation, early in the exponential phase of growth. The early appearance of ethanol indicated that it may be useful as a marker for the rapid detection of *E. coli*.

Urine test system for distinguishing *E. coli* and *Proteus* spp. Urine varies in composition and in ability to support bacterial growth. Since the proposed test was to involve bacterial growth, the urine test medium was enriched with PW and a phosphate buffer, pH 7.2. In addition, lactose and methionine were incorporated into the medium as substrates for the specific products produced by *E. coli* (ethanol) and *Proteus* spp. (dimethyl disulfide).

In a simulated urine test, a volume of filter-sterilized urine was added to an equal volume of urine test medium ingredients in various combinations, inoculated with about 10⁶ organisms per ml of *E. coli* and/or *P. mirabilis*, incubated still for 18 h, and examined for optical density and by HS-GLC (Table 4). Growth of both organisms was poor in buffered urine alone, confirming the necessity for added nutrients. *E. coli* appeared to be inhibited by methionine and stimulated by lactose, but the growth of *P. mirabilis* was unaffected by either substrate. *P. mirabilis* formed large amounts of dimethyl disulfide from the methionine media, and *E. coli* did not form any dimethyl disulfide. *E. coli* formed large amounts of ethanol and small amounts of *n*-propanol from the lactose media. *P. mirabilis* did not form any alcohol, but it did produce a small amount of ethyl methyl ketone, which has a slightly shorter retention time than ethanol. Both dimethyl disulfide and ethanol were detected from the complete medium incubated with a mixture of *E. coli* and *P. mirabilis*.

The results in Table 4 and the chromatograms of *P. mirabilis* and *E. coli* in the com-

FIG. 1. Ethanol and *n*-propanol production by *E. coli* from still cultures in lactose PW. Symbols: ○, viable count; ●, ethanol; ■, *n*-propanol.

plete medium (Fig. 2) show that this urine test system clearly distinguished *E. coli* and *P. mirabilis*. A compound at retention time of 1 min was present in large amounts in the urine alone and in all of the test-medium combinations (Table 4). The compound was not identified but was probably an amine because it had a fishy odor and tailed markedly on the Carbowax col-

TABLE 4. Volatile products formed in 18 h from urine test media by still cultures of *E. coli* and *P. mirabilis*

Medium	Culture	Optical density	Peak area (cm ²)			Peak ht of amine (cm) ^a
			Dimethyl disulfide	Ethanol	<i>n</i> -Propanol	
Urine + buffer	<i>E. coli</i>	0.28	0	0	0	500
	<i>P. mirabilis</i>	0.16	0	0	0	258
Urine + buffered PW	<i>E. coli</i>	0.75	0	0	0	484
	<i>P. mirabilis</i>	0.34	3	0	0	310
Urine + buffered methionine PW	<i>E. coli</i>	0.55	0	0	0	280
	<i>P. mirabilis</i>	0.34	850	0	0	438
Urine + buffered lactose PW	<i>E. coli</i>	1.25	0	636	11	324
	<i>P. mirabilis</i>	0.35	3	0	0	376
Urine + buffered methionine lactose PW	<i>E. coli</i>	0.82	0	591	10	290
	<i>P. mirabilis</i>	0.35	400	0	0	328
	<i>E. coli</i> + <i>P. mirabilis</i>	— ^b	144	1620	18	465

^a Area not determined because of long tailing (Fig. 2).^b —, Not recorded.

FIG. 2. Chromatograms of HS products from still cultures of *P. mirabilis* and *E. coli* in urine + buffered lactose methionine PW incubated for 18 h at 37°C. GLC stationary-phase material, Carbowax 20M; column temperature, 100°C; attenuation setting, $\times 10$ unless otherwise marked. Peak a, unidentified product, probably an amine; b, ethyl methyl ketone; c, dimethyl disulfide; d, ethanol; e, *n*-propanol.

um (Fig. 2). The presence of methyl mercaptan at retention time 0.95 min could not be assessed since it was masked by the large unidentified peak.

Correlation between size of inoculum of *E. coli* or *P. mirabilis* in urine test system and rate of product formation. In a time-course study, the complete urine test medium mixed

with an equal volume of sterile urine was inoculated in duplicate with an overnight culture of *E. coli* in urine to give concentrations of about 10^3 , 10^4 , 10^5 , and 10^6 organisms per ml and incubated still, and the products produced were examined by HS-GLC. A similar experiment was done with *P. mirabilis*, except that cultures were incubated shaking. An *E. coli* control for this experiment was inoculated with about 10^6 organisms per ml.

Ethanol was first detected at 4 h in still cultures with an *E. coli* inoculum of 10^6 organisms per ml, and at 6 h, cultures with initial inocula of 10^5 and 10^4 organisms per ml could easily be distinguished from cultures with smaller inocula (Fig. 3). Dimethyl disulfide was first detected at 3 h with a *P. mirabilis* inoculum of 10^6 organisms per ml, and at 4 h, cultures with initial inocula of 10^5 and 10^6 organisms per ml could easily be distinguished from cultures with smaller inocula (Fig. 4). The urine used for this experiment did not yield the unidentified product encountered in the experiments shown in Table 4 and Fig. 2, so that methyl mercaptan was not masked. Methyl mercaptan was first detected at 4 h from 10^6 organisms per ml and at 5 h from 10^5 organisms per ml. The *E. coli* control culture formed a small amount of dimethyl disulfide at 6 h (peak area, 25 cm²), less than the *P. mirabilis* culture with an initial inoculum of 10^3 organisms per ml (peak area, 40 cm²).

It was concluded that significant numbers of *E. coli*, 10^5 or more per ml, could be detected in urine by ethanol production from lactose in about 5 h, and significant numbers of *Proteus* spp. could be detected by dimethyl disulfide production from methionine in about 4 h and by methyl mercaptan production in about 5 h.

DISCUSSION

It appears probable that the HS-GLC system will detect and distinguish significant numbers of *E. coli* and *Proteus* spp. rapidly.

The use of GLC to detect metabolic products of bacteria and viruses as a means of rapid diagnosis has been proposed (1, 2, 8-10) for the examination of biological fluids during the course of infection in the living host animal. The present HS-GLC method tests for metabolic products in a controlled situation by culturing the biological specimen in a medium with specific substrates from which it has been shown that particular organisms form specific products. The biological specimen toward which the work has been directed is urine, but it could be applicable wherever *E. coli* or *Proteus* spp. might be found. Urine usually con-

FIG. 3. Relationship between size of inoculum of *E. coli* and rate of ethanol production from still cultures in urine + buffered lactose methionine PW. Inoculum: ■, 10^6 *E. coli*/ml; ●, 10^5 *E. coli*/ml; ▲, 10^4 *E. coli*/ml; ○, 10^3 *E. coli*/ml.

tains a mixture of bacteria, and the method proposed here runs counter to a bacteriological principle, that tests for metabolic products are valid only when performed with pure cultures. However, the pathogen is usually in pure culture in the urinary bladder and, in well-taken specimens, greatly outnumbers contaminants added during voiding. It is probable that, since tests are done after short incubation, only the predominant organism will be detected.

The simulated tests gave warning that naturally occurring substances in urines might interfere with the test in practice, either by masking specific products or by stimulating their rate of production to a variable extent. A substance in one urine masked methyl mercaptan by coeluting with it. Methyl mercaptan is secondary to dimethyl disulfide as a marker for

FIG. 4. Relationship between size of inoculum of *P. mirabilis* and rate of dimethyl disulfide and methyl mercaptan production from shaken cultures in urine + buffered lactose methionine PW. Inoculum: ■, 10^6 *P. mirabilis*/ml; ●, 10^5 *P. mirabilis*/ml; ▲, 10^4 *P. mirabilis*/ml; ○, 10^3 *P. mirabilis*/ml; ■--■, 10^6 *E. coli*/ml.

Proteus spp., but if test urines contained compounds that coeluted with ethanol or dimethyl disulfide, these specific products would not be useful criteria for the presence of *E. coli* and *Proteus* spp., and the test would be invalidated. The test depends on rates of product formation to indicate sizes of inocula. When the large amounts of ethanol formed from lactose by *E. coli* in medium containing urine (Table 4) are compared with the much smaller amounts from the same medium without urine (Table 2), it is apparent that this particular urine contained a substance(s) that stimulated the yield of ethanol in 18 h by this strain of *E. coli* and might also have increased the rate of ethanol production.

The HS-GLC urine test system appears to be promising, but a trial of the test on a large number of urines from infected and uninfected patients is needed to show whether contaminants added during voiding or naturally occurring compounds in urines cause false results.

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