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Assessment of Technique for Rapid Detection of *Escherichia coli* and *Proteus* Species in Urine by Head-Space Gas-Liquid Chromatography

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A test depending on the production of ethanol by *Escherichia coli* from lactose and dimethyl disulfide by *Proteus* spp. from methionine in the early exponential phase of growth and the detection of these products by head-space gas-liquid chromatography has been applied to 75 specimens of urine selected to provide the most stringent trial of the test. The test was found to be rapid and reliable for the commonest findings in the microbiological examination of urine. In 3 to 4 h it detected "significant" numbers ($>10^5$/ml) of *E. coli* or of *Proteus mirabilis* or *P. inconstans* A, identified as *Proteus* spp., in 23 urines. It recorded the absence of infection from 32 urines containing borderline or "not significant" numbers of any organism. Significant numbers of other organisms in 13 urines were not mistaken for *E. coli* or *Proteus* spp. However, the test was less successful for some less common findings. *Klebsiella ozaenae* in significant numbers in one urine was mistaken for *E. coli*. *P. morganii* in significant numbers in one urine was not detected. *E. coli* or *P. mirabilis* mixed with significant numbers of another organism were not detected in four out of five urines. The technique is simple and could be automated. It appears to merit more extensive trial in a hospital laboratory and further development to detect and correctly identify more species that cause urinary tract infections.

A urine test system based on head-space (HS) gas-liquid chromatography (GLC; HS-GLC) to detect ethanol and dimethyl disulfide from lactose-methionine medium cultures of the bacteria in urine has been proposed as a rapid method for detecting and identifying significant numbers (2, 6) of *Escherichia coli* and *Proteus* spp. in urine (4). In the present investigation the urine test system has been applied to urines from infected and uninfected patients selected with a bias to make the trial as searching as possible. The investigation was designed to test in practice whether any infecting organisms other than *E. coli* or *Proteus* spp. in urine would be confused with these species, and whether contaminants could be distinguished from significant numbers by an appropriate choice of incubation period. It was also necessary to determine whether naturally occurring compounds in urine would mask either ethanol or dimethyl disulfide by coeluting with them or would affect the rate of product formation by significant numbers of *E. coli* or *Proteus* spp. and invalidate the quantitative nature of the test. The parameters that would give the most reliable results were examined by incubating quadruplicate cultures either shaken or still and for either of two periods of time, before testing for ethanol and dimethyl disulfide by HS-GLC.

MATERIALS AND METHODS

Urine samples. Specimens of urine were obtained from 75 hospital patients, stored at 4°C, and tested, usually on the day of collection. Patients from whom urine specimens were examined were selected in consultation with the hospital bacteriologist. Limits were placed on the numbers of urines containing either significant numbers of *E. coli* or minimal numbers of organisms of any kind, and as many urines as possible containing either significant numbers of organisms other than *E. coli* or not significant but borderline numbers of any species were selected. The bacteriological findings in this paper are not typical of the whole range of urine specimens in the hospital but were selected to provide the most stringent trial of the test.

Culture media. The urine test medium was prepared by dissolving 3 g of L-methionine (0.2 M), 2 g of lactose, and 2 g of peptone in 100 ml of 0.2 M potassium dihydrogen phosphate-disodium hydrogen phosphate buffer at pH 7.2 and was sterilized by filtration. The medium was dispensed in 5-ml samples to Universal bottles, which were either screw capped or loosely plugged with cotton wool.

MacConkey agar (Oxoid) was prepared as directed by the manufacturers. Firm blood agar containing

7.5% equine blood and 3.6% agar was prepared from nutrient broth (Oxoid no. 2).

Urine cultures. Viable counts (7) were performed on 10-fold dilutions of urine, each count being a mean of 9 drops, 3 on each of three well-dried MacConkey plates incubated overnight at 37°C. The number of colonies counted was between 45 and 450, except when there were fewer in undiluted urine. If more than one colony type could be distinguished, each was counted and recorded separately. A loopful of urine was plated on firm blood agar and incubated for 48 h at 37°C to detect organisms that failed to grow on MacConkey agar and to assist in the isolation of pure cultures. All isolates were identified by standard tests (3).

GLC. The preparation of HS samples, GLC conditions, and calculations of peak areas were as described previously (5); 4.8 g of anhydrous $MgSO_4$ sometimes replaced K_2CO_3 to prepare HS samples. Peak areas less than 1.5 cm^2 for ethanol and less than 2 cm^2 for dimethyl disulfide were recorded as trace. When small volumes of 2 or 3 ml were used to prepare HS samples, proportionately smaller glass serum vials and less K_2CO_3 were used, but the volume of HS gas sample was unchanged (0.5 ml).

Method of the test. A 5-ml portion of a patient's urine was added to each of two screw-capped and two loosely plugged bottles of urine test medium. The final concentration of methionine was 0.1 M and that of lactose was 1%. The screw-capped bottles were incubated still, and the loosely plugged bottles were shaken on a rotary shaker at 160 rpm at 37°C. Samples (8 ml) of urine cultures were examined by HS-GLC after incubation for either 3 or 4 and 5 h, and an 8-ml sample of undiluted and unincubated urine was examined by HS-GLC as a control.

RESULTS

Effect of using smaller volumes for the test. On 10 occasions small volumes of culture and urine were used to prepare HS samples. The results were similar to those from the usual 8-ml volume.

Undiluted and unincubated urine controls. About one-third of the urine controls yielded GLC peaks with the same retention time as ethanol, with areas ranging from 0.6 to 50 cm^2. When these were observed, appropriate corrections were made to the areas of ethanol peaks from urine cultures.

More than half of the urine controls yielded moderate amounts of substances detectable by HS-GLC. These differed from urine to urine, but some that eluted early appeared to be amines, by smell and because they were suppressed when $MgSO_4$, an acid salt, replaced K_2CO_3, an alkaline salt, for preparing HS samples. Ethanol, dimethyl disulfide, and methyl mercaptan were released into HS by $MgSO_4$.

Time of incubation of urine cultures. The first urine cultures were examined by HS-GLC after incubation for 4 and 5 h, because results from the development of the test appeared to show that significant numbers of *E. coli* would be detectable by ethanol production in still cultures in 5 h and significant numbers of *Proteus* spp. would be detectable by dimethyl disulfide production in shaken cultures in 4 h. However, when 19 urines, including 7 containing significant numbers of *E. coli*, had been examined (Table 1), it was clear that ethanol production was already substantial in 4 h. Consequently, the remaining 56 urine cultures were examined after incubation for 3 and 5 h.

Urines containing significant numbers of *E. coli* or *P. mirabilis* (Table 1). Ethanol production in 3 h was sufficient for detection of *E. coli*, particularly in still cultures. There was always more ethanol in cultures than in controls. Dimethyl disulfide was always detectable in 3-h shaken cultures. However, it was not detectable in six of the nine 3-h still cultures, and only trace amounts of it were detected in still cultures even after 5 h. No methyl mercaptan was detected even in the 5-h shaken cultures. It was concluded that 3-h still cultures indicated significant numbers of *E. coli* most quickly and plainly and that it was unlikely that the incubation time could be shortened. Small amounts of *n*-propanol, eluting after ethanol and before dimethyl disulfide, were produced by all still cultures.

Dimethyl disulfide production in 3 h was sufficient for detection of *P. mirabilis*, particularly in shaken cultures. No dimethyl disulfide was detected in controls. Yields of dimethyl disulfide were more in both still and shaken cultures than in any culture of urine containing significant numbers of *E. coli*. Methyl mercaptan was detected in only one 3-h still culture, but it was detected in three 3-h and five 5-h shaken cultures. Only small amounts of ethanol were detected even after 5 h. No *n*-propanol was detected. It was concluded that the 3-h still cultures chosen for *E. coli* would indicate significant numbers of *P. mirabilis*. However, shaken cultures indicated *P. mirabilis* more plainly, and further trials would probably show that *P. mirabilis* could be detected in less than 3 h in shaken cultures.

Urines containing borderline or "not significant" numbers of organisms (Table 2). Five of the urines with viable counts of 10^4 to 10^5 of various species per ml, including *E. coli* and *P. mirabilis*, did not yield any detectable ethanol or dimethyl disulfide even after incubation for 5 h. However, four urines with mixed growths, including *P. mirabilis*, yielded some dimethyl disulfide after 5 h, particularly in the shaken

TABLE 1. *Yields of ethanol and dimethyl disulfide from cultures of urine containing significant numbers of E. coli or P. mirabilis*

Infecting bacterium	No. of specimens	Viable counts	Avg peak area (cm²)							
			Ethanol				Dimethyl disulfide			
			Shorter incubation (3 h)		Longer incubation (5 h)		Shorter incubation (3 h)		Longer incubation (5 h)	
			Still	Shaken	Still	Shaken	Still	Shaken	Still	Shaken
E. coli	7	4.4×10^6–2.0×10^8	219[a]	144[a]	283	255	Tr[a]	31[a]	Tr	54
			45–339[b]	23–284	90–542	90–488		5.8–89		13–90
	9	6.9×10^6–3.1×10^8	90	86	282	256	Tr	14	Tr	54
			40–174	0–181	135–503	89–436		1.3–29		21–77
P. mirabilis	6	5.0×10^5–1.3×10^8	tr	2.5	4.6	1.6	45	363	119	860
				0–14	0–61	0–50	8–113	116–613	19–290	258–1290

[a] 4-h incubation.
[b] Range of peak areas.

cultures, and a product with the same retention time as ethanol from shaken cultures of one of them.

Similar results were obtained from 23 urines with counts of less than 10^4 of various species per ml. Neither ethanol nor dimethyl disulfide was detected from 22 of them, but one urine with 10^3 of both *E. coli* and *P. mirabilis* per ml yielded some dimethyl disulfide.

Dimethyl disulfide is a more readily detectable indicator of *P. mirabilis* than ethanol is of *E. coli*. It is necessary to use the shortest incubation time that is satisfactory for *E. coli* to avoid detection of dimethyl disulfide from cultures of numbers of *P. mirabilis* that are not significant.

Significant numbers of organisms not *E. coli* or *P. mirabilis* (Table 3). The result from one specimen containing significant numbers of *Klebsiella ozaenae* was indistinguishable from urines containing significant numbers of *E. coli* even to the detail of some *n*-propanol in still cultures. However, two specimens containing significant numbers of *Klebsiella aerogenes* (*Klebsiella pneumoniae*) yielded only small amounts of ethanol. One of them yielded a little *n*-propanol at 5 h, but they would not have been mistaken for *E. coli*. Tests of 4-h still cultures in lactose-methionine-peptone water of the strains of *K. ozaenae* and *K. aerogenes* isolated from these urines confirmed that *K. ozaenae* gave a high yield of ethanol, but none was detected from *K. aerogenes*.

Cultures of urines containing significant numbers of *Serratia marcescens*, *Streptococcus faecalis*, mixtures in which *S. faecalis* or *Aerococcus viridans* was predominant, or significant numbers of *Candida albicans* yielded small amounts of ethanol, but none would have been mistaken for *E. coli*. One urine containing a mixture including borderline numbers of *P. mirabilis* yielded dimethyl disulfide from shaken cultures, but still cultures would not have been mistaken for significant *P. mirabilis*.

The result from *Proteus inconstans* A was indistinguishable from *P. mirabilis*. This was expected from the study of methionine catabolism by *Proteus* spp. (5), but the slow response of *P. morganii* was unexpected. It would have been mistaken for numbers of *P. mirabilis* that were not significant.

Neither ethanol nor dimethyl disulfide was detected from the five urines with significant numbers of *Acinetobacter anitratus*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, or a *Corynebacterium* sp. Both *Staphylococcus* cultures and one *A. anitratus* were lactose fer-

TABLE 2. *Yields of ethanol and dimethyl disulfide from cultures of urine containing borderline and not significant numbers of any organism*

Type of bacterial count	No. of specimens	Viable counts		Peak area (cm^2)							
				Ethanol				Dimethyl disulfide			
				3- to 4-h incubation		5-h incubation		3- to 4-h incubation		5-h incubation	
				Still	Shaken	Still	Shaken	Still	Shaken	Still	Shaken
Borderline, 10^4–10^5/ml	5	1.1×10^4–7.8×10^4		0	0	0	0	0	0	0	0
	1	*S. faecalis*,	6.0×10^4	0	0	0	0	0	0	0	129
		P. mirabilis,	2.1×10^4								
	1	*S. faecium*,	2.3×10^4	0	0	0	0	0	0	2.6	9.7
		K. aerogenes,	8.0×10^3								
		P. mirabilis,	1.0×10^3								
	1	*P. mirabilis*,	2.6×10^4	0	0	0	0	0	0	26	142
		S. epidermidis,	6.7×10^2								
	1	Enterococcus,	4.3×10^4	tr	8.0	tr	25	0	0	40	452
		Corynebacterium sp.,	6.3×10^3								
		P. mirabilis,	7.7×10^3								
		Micrococcus,	2.0×10^3								
Not significant, $<10^4$/ml	22	0–8.2×10^3		0	0	0	0	0	0	0	0
	1	*E. coli*,	2.3×10^3	0	0	0	0	0	0	13	123
		P. mirabilis,	1.0×10^3								
		Enterococcus,	3.0×10^2								

TABLE 3. *Yields of ethanol and dimethyl disulfide from cultures of urines containing significant numbers of organisms not E. coli or P. mirabilis*

Organisms	Viable counts	Peak area (cm^2)							
		Ethanol				Dimethyl disulfide			
		3- to 4-h incubation		5-h incubation		3- to 4-h incubation		5-h incubation	
		Still	Shaken	Still	Shaken	Still	Shaken	Still	Shaken
K. ozaenae	5.2×10^8	525	78	1220	344	0	32	0	71
K. aerogenes	1.1×10^6	0	0	2.0	0	0	0	0	2.6
K. aerogenes	8.3×10^5	5.0	0	9.0	0.9	0	0	0	10
S. marcescens	1.3×10^7	19	9.9	23	36	0	0	0	0
S. faecalis	2.7×10^6	8.5	0	31	2.0	0	0	0	0
S. faecalis	7.1×10^5								
K. aerogenes	4.9×10^4	tr	tr	15	7.2	0	0	0	0
E. coli	1.5×10^3								
S. faecalis	4.1×10^6								
E. coli	2.6×10^4	0	0	23	3.0	tr	0	0	0
A. viridans	7.2×10^7								
E. coli	5.7×10^4								
S. epidermidis	5.0×10^4	24	8	48	0	tr	61	tr	1,160
P. mirabilis	2.3×10^4								
C. albicans	1.8×10^5	5.5	2.0	19	5	0	0	0	0
P. inconstans A	4.9×10^7	7.6	0	6.3	13	90	306	161	390
P. morganii	3.9×10^5	0	0	0	0	0	0	194	335
A. anitratus	7.4×10^5	0	0	0	0	0	0	0	0
S. epidermidis	6.1×10^6	0	0	0	0	0	0	0	0
S. aureus	1.6×10^6	0	0	0	0	0	0	0	0
A. anitratus	1.1×10^6								
K. aerogenes	4.5×10^3	0	0	0	0	0	0	0	0
Corynebacterium sp.	1.2×10^6								
S. faecalis	4.1×10^4	0	0	0	0	0	0	0	0
P. mirabilis	5.0×10								

menters in conventional tests.

Significant numbers of more than one organism including *E. coli* or *P. mirabilis* (Table 4). Ethanol production by *E. coli* in four urines in which it was outnumbered by another organism was markedly suppressed in both 3 and 5 h. *E. coli* was predominant in a fifth urine and was clearly apparent in 4 h. Dimethyl disulfide production by *P. mirabilis* in two urines in which it was predominant was clearly apparent in 3-h shaken cultures. However, *P. mirabilis* was not detected in 3-h still cultures of one of the urines. The other patient was oliguric, and the specimen of urine was not large enough for four tests and a control. The 3-h still culture was omitted, but the high yield from the 5-h still culture suggests that *P. mirabilis* would have been detected in 3 h.

Production of specific products by mixtures of *E. coli* and *P. mirabilis*. The failure to detect *E. coli* in some mixed infections was unexpected. An artificial mixture of approximately equal numbers of *E. coli* and *P. mirabilis* yielded both ethanol and dimethyl disulfide in 18 h in a simulated test, there being an increase in ethanol and a decrease in dimethyl disulfide compared with each culture incubated separately (Table 4 in reference 4). Two factors may have contributed to the different result. The time of incubation was 18 h in the simulated urine test and only 3 to 5 h in the tests on infected urines. *E. coli* was mixed with almost equal numbers of *P. mirabilis* in the simulated test but was out-numbered in all four urines in which it was not detected. It was detected in the fifth urine, in which it was predominant.

Mixtures of *E. coli* and *P. mirabilis* were tested using the strains from the urine in which they occurred in the proportion of 5.5×10^5 to 1.1×10^7 per ml (Table 4). In a simulated test, filter-sterilized urine was mixed with urine test medium, inoculated with *E. coli* and *P. mirabilis* either alone or as mixtures, and incubated still for both 3 and 18 h (Table 5). When *E. coli*

TABLE 4. *Yields of ethanol and dimethyl disulfide from cultures of urines containing significant numbers of more than one organism including E. coli or P. mirabilis*

Organism	Viable counts	Peak area (cm^2)							
		Ethanol				Dimethyl disulfide			
		3- to 4-h incubation		5-h incubation		3- to 4-h incubation		5-h incubation	
		Still	Shaken	Still	Shaken	Still	Shaken	Still	Shaken
E. coli / *K. aerogenes*	2.0×10^8 / 1.3×10^5	218	82	139	240	0	13	0	57
P. mirabilis / *E. coli*	1.1×10^7 / 5.5×10^5	tr	0	29	0	tr	516	177	839
P. mirabilis / *E. coli*	3.6×10^7 / 1.3×10^5	0		18	0		1,030	710	1,160
K. aerogenes / *E. coli*	3.1×10^7 / 1.0×10^5	12	0	39	27	0	3.9	0	6.5
P. fluorescens / *E. coli*	2.9×10^6 / 2.3×10^5	0	0	0	0	0	0	0	0

TABLE 5. *Effects of proportions of E. coli and P. mirabilis in mixtures and of time of incubation on yields of specific products from still cultures*

Mixtures (no. of viable organisms/ml)		Time of incubation (h) and yield of products (peak areas in cm^2)			
E. coli	*P. mirabilis*	3		18	
		Ethanol	Dimethyl disulfide	Ethanol	Dimethyl disulfide
1.6×10^7	0	168	0	525	0
1.6×10^6	0	90	0	392	0
1.6×10^5	0	39	0	450	0
1.6×10^7	3.1×10^5	300	0	600	56
1.6×10^6	3.1×10^5	90	tr	600	45
1.6×10^6	3.1×10^6	42	tr	850	144
1.6×10^5	3.1×10^6	18	11	1,050	200
1.6×10^5	3.1×10^7	9	8	750	480
0	3.1×10^5	0	8	0	400
0	3.1×10^6	0	31	0	340
0	3.1×10^7	0	94	0	395

was outnumbered by *P. mirabilis* ethanol production was suppressed in 3 h. In 18 h, ethanol production was stimulated in all mixtures of *E. coli* with *P. mirabilis*. In 3 h, dimethyl disulfide production by *P. mirabilis* was suppressed by *E. coli* in all mixtures, and in 18 h, dimethyl disulfide production was suppressed except when *P. mirabilis* was predominant. Both incubation time and the relative proportions of *E. coli* and *P. mirabilis* in mixtures affect results.

Summary of results. In the trial of the urine test system the 3-h still cultures gave the correct results for 69 out of 75 clinical specimens. Twenty-three containing significant numbers of *E. coli* (16) or *Proteus* spp. (7) and 32 containing numbers of organisms that were not significant were correctly reported; 13 containing significant numbers of organisms, not *E. coli* or *Proteus* spp., were not mistaken for either *E. coli* or *Proteus*; and *E. coli* was detected in one urine containing both *E. coli* and *K. aerogenes* in significant numbers. However, significant numbers of *K. ozaenae* were once mistaken for *E. coli*; significant numbers of *P. morganii* were once not detected; and *E. coli* or *P. mirabilis* were not detected in four urines containing significant numbers of more than one organism.

DISCUSSION

The HS-GLC urine test performed better in the natural situation than was expected from the simulated trials (4). When either *E. coli* or *Proteus* spp. were growing in medium containing the urine in which they occurred naturally, their specific products were more readily detected, and the time of incubation could be shortened from 4 or 5 h to 3 h, a significant improvement in the speed of the test. At 3 h, "significant" numbers of *E. coli* or *Proteus* spp.

could be distinguished from borderline or not significant numbers of any organism. Significant numbers of other organisms were not mistaken for *E. coli* or *Proteus* spp., apart from one infection with *K. ozaenae*. It was expected that some lactose-fermenting enterobacteria such as *K. ozaenae* would be as active as *E. coli* in producing ethanol from lactose (4), and it would be desirable either to have some means of distinguishing them from *E. coli*, or to develop a more specific marker for *E. coli*. Alternatively, if arabinose replaced lactose in the test, a wider range of enterobacteria would probably produce ethanol and be detected (4). The advantage of detecting more infections might outweigh the loss of precision in naming the causes of them.

P. morganii was undetected in 3-h cultures, possibly because of slower growth, a characteristic that was noted in earlier experiments (5). A test medium that satisfied the growth requirements of more exacting organisms would be an improvement.

In the natural situation the test performed poorly for urines containing significant numbers of more than one organism, i.e., mixed infections. Apparently it is unusual for naturally occurring mixtures incubated for only 3 h to yield products as if each element of the mixture were growing alone. In particular, the organism that is in the minority is suppressed.

The rapid production of large amounts of dimethyl disulfide and methyl mercaptan from methionine that marks *Proteus* spp. has not been observed with any other organism encountered in the trial and so far appears to be a valid discriminator of the genus in the context of urinary tract infection. Compounds in urine sometimes coelute with methyl mercaptan, a problem that did not arise with dimethyl disulfide and ethanol. This problem and the appearance of methyl mercaptan later in growth than dimethyl disulfide make the latter the primary marker for *Proteus* spp. However, $MgSO_4$ appears to suppress the appearance in HS of some compounds in urine that coelute with mercaptan. Although $MgSO_4$ is less effective in releasing ethanol and dimethyl disulfide in HS (5), it might be preferred to K_2CO_3 under the conditions that apply in practice.

Growth-stimulating substances in some urines may have had a variable effect on the rate of specific product formation, but the appearance of either ethanol or dimethyl disulfide after a short (3 h) incubation remained valid as an indicator of significant numbers of *E. coli* or *Proteus* spp. in urine.

Although several kinds of patient with urinary tract infection do not excrete as many as 10^5 organisms per ml (1), there is a large group of patients to whom this criterion for distinguishing significant bacteriuria appears to apply. It is for this group that the rapid urine test is meant to be used. The test appears to be successful in detecting the commonest findings in such patients.

A trial of the method using a control and only one test, say a 3-h still culture, in parallel with routine methods in a diagnostic laboratory is indicated. The discovery of products, detectable in the same HS-GLC system, that would be markers for other bacteria that commonly cause urinary tract infections such as *Staphylococcus saprophyticus* (8), *P. aeruginosa*, or *S. faecalis* could be undertaken to enlarge the scope of the test. At this stage the method cannot be seen as a replacement for full examination of urine by microscopy and culture. However, it is simple and quick. Machines for fully automated HS-GLC analysis are available, having been developed for the determination of ethanol in blood and urine (9). The test may be used as a rapid method to give an early warning of infection in selected high-risk patients and as an automated method for specimens that ordinarily would not be cultured. If bacteriostatic concentrations of antibiotics in parallel cultures were shown to suppress product formation, sensitivity tests could be incorporated in the rapid method.

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