

Isolation of Salmonellae and Shigellae from an Artificial Mixture of Fecal Bacteria¹

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Numerous selective media, available commercially, act by suppressing "normal" bacterial inhabitants of the intestine while permitting the growth of so-called pathogenic representatives of the family *Enterobacteriaceae*. This investigation attempts to evaluate the action of *Salmonella-Shigella* (SS) agar, xylose lysine desoxycholate (XLD) agar, and hektoen enteric (HE) agar. Salmonellae and shigellae, isolated from clinical material, were mixed in various ratios with escherichiae, *Klebsiella-Enterobacter-Serratia* group bacteria, and members of the tribe *Proteeae*, also of clinical origin. Several of the mixtures were plated in multiple dilutions on the three media. Stools in preservative were also used for evaluation of the media after the addition of definite numbers of the pathogenic bacteria. Results indicate that SS agar suppresses the shigellae along with the autochthonous members of *Enterobacteriaceae*. XLD and HE agars readily permit the recovery of shigellae as well as salmonellae. This recovery is not obscured by the higher yield of other species obtained with these media.

The need to rapidly recognize salmonellae and shigellae in patients and their contacts, as well as in suspect foods, has led to the introduction of several selective media. These recent additions are alleged to increase the ease of sequestering bacteria of clinical significance from the great number of representatives of the family *Enterobacteriaceae* usually encountered in fecal specimens. This study was initiated to investigate the performance of xyline lysine desoxycholate (XLD) agar and hektoen enteric (HE) agar vis-a-vis an established selective medium, *Salmonella-Shigella* (SS) agar. In an attempt to bring a measure of standardization to this problem, artificial mixtures of various enteric bacteria were used. This report is a summary of the findings.

MATERIALS AND METHODS

Microorganisms. The bacteria used are listed in Table 1. All organisms were isolated from clinical material. Identification of salmonellae and shigellae was confirmed by the Salmonella Center of Beth Israel Hospital, New York, N.Y., through the courtesy of J. Winter.

Media. Besides XLD, prepared as directed by Taylor (10), HE [Pfizer Diagnostic (5)], SS (BBL), Trypticase Soy Agar (TSA; BBL) and, occasionally, Eosin-Methylene Blue (EMB) agar (BBL) served as solid media controls. Brain Heart Infusion broth

(BHI; BBL) was used for the preparation of the bacteria for artificial mixtures as well as the diluent. Stool preservative as described by Kauffmann (4), in 5-ml volumes, served as the suspending medium for control stools.

Artificial mixtures. The test and diluent bacteria were grown in BHI broth for 18 hr at 35 C. Dilutions of the test bacteria were made with the diluent microorganisms to achieve dilutions of 1:100, 1:1000, and 1:10,000. Thus, test bacteria in broth were diluted to achieve concentrations of 10^5 , 10^4 , and 10^3 organisms/ml, with broths containing 10^7 diluent bacteria/ml. The test bacteria were tested singly and in combination against single and multiple combinations of diluent bacteria. The artificial mixtures were then diluted further with BHI broth to final total concentrations of approximately 10^5 and 10^4 total organisms/ml. The selective media and the control agar were inoculated after mechanical agitation on a Vortex Genie (Scientific Industries, Inc., Springfield, Mass.) with 0.05-ml volumes via sterile pipettes, followed by spreading with a bacteriological loop. All tests were conducted in duplicate and were repeated several times. Dilutions of test bacteria with uninoculated BHI broth were also tested. All mixtures and controls were applied to the enrichment media and TSA, the latter intended to give a measure of the viable organisms in the mixture.

A group of 50 stools in 5.0 ml of stool preservative, screened to ascertain the absence of salmonellae and shigellae, was supplemented with 10^2 , 10^3 , and 10^4 salmonellae or shigellae, or both, contained in 0.1-ml volumes. These specimens were then plated on the various media as well as on EMB agar with an 0.02-ml loop and were read after 18 hr at 35 C.

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TABLE 1. *Artificial mixtures in Brain Heart Infusion broth*

Test bacteria	Diluent bacteria
1. <i>Salmonella typhimurium</i>	1-3. <i>Escherichia coli</i> (3)
2. <i>S. infantis</i>	4. <i>Klebsiella</i> sp.
3. <i>S. newport</i>	5. <i>Enterobacter aerogenes</i>
4. <i>Shigella sonnei</i>	6. <i>Proteus mirabilis</i>
5. <i>S. dysenteriae</i>	7. <i>P. morgani</i>

RESULTS

Results obtained with the salmonellae are summarized in Table 2. No significant variation was observed when different serotypes of the genus were mixed with escherichiae isolated from different individuals. The use of other members of the family *Enterobacteriaceae*, i.e. tribes *Proteeae* or *Klebsielleae*, as diluent bacteria did not essentially alter the ratios recovered from the artificial mixtures of bacteria. As would be expected, the ratio of test to diluent bacteria and to the final dilution cultured was manifested in absolute terms. More salmonellae were recovered when their presence in the mixtures was proportionally greater. When the ratio of salmonellae to diluent bacteria exceeded 1:10³ and the final concentration of bacteria was reduced to 10⁴ per ml, recovery of salmonellae was difficult. It depended on the chance presence of these bacteria in a mixture numerically reduced to the point of their dilution with the other organisms. The three enrichment media displayed sensitivity of the same order of magnitude toward the salmonellae, whereas their selectivity for these organisms differed. Thus, SS agar con-

sistently suppressed the diluent bacteria. The selectivity of XLD and HE agars was much less evident. Although some retardation of the diluent bacteria was manifested by both agars, as demonstrated by the failure of the 10⁴ final concentration of bacteria per ml to yield even 100 colonies per plate, the selectivity did not approach that displayed by SS. Despite a uniformly higher recovery rate of diluent bacteria with HE agar, the recovery was of the same order of magnitude as with XLD agar.

The results with shigellae as test bacteria are presented in Table 3. Here, also, the nature of the diluent bacteria, whether present singly or in combination, exerted no real effect on the recovery of the test organisms. The species or serogroup of *Shigella* used did not exert any influence on these results as well. The dilution effects noted for the salmonellae also apply to the shigellae. The selectivity of SS agar vis-a-vis the shigellae, noted by others, especially Taylor (8), is very obvious and compares with the suppression of other members of the family *Enterobacteriaceae*. On the other hand, XLD and HE agars permitted the recovery of shigellae with equal ease and with negligible differences in terms of absolute numbers.

It was noted during the study of these artificial mixtures that reproducible results were obtained only if the mixtures were tested within the first 30 min after combination. Salmonellae and shigellae were added, therefore, to 50 actual stools in stool preservative which had been found free of representatives of both test bacteria. These triplicate specimens were then kept at room temperature for 8 hr before final mixing on a Vortex Genie and

TABLE 2. *Isolation of salmonellae from artificial mixtures with escherichiae*^a

Bacterial mixture	Ratio of <i>Salmonella</i> to <i>Escherichia</i>	Final bacterial concn/ml cultured	XLD bacteria		HE bacteria		SS bacteria		TSA bacteria
			Diluent	Test	Diluent	Test	Diluent	Test	
<i>Salmonella</i> and <i>Escherichia</i>	1:10 ²	10 ⁵	>300	70	>300	72	41	62	>300
		10 ⁴	65	11	85	9	3	14	>300
	1:10 ³	10 ⁵	>300	23	>300	19	58	31	>300
		10 ⁴	59	8	81	7	7	6	>300
	1:10 ⁴	10 ⁵	>300	2	>300	4	37	5	>300
		10 ⁴	63	0	74	0	11	1	>300

^a Values represent colonies counted after incubation for 18 hr at 35 C. Results obtained with the various salmonellae and escherichiae did not exceed the variations manifested by each strain when tested repeatedly. Colony counts represent the average obtained with all tests performed in duplicate and repeated 7 to 10 times. Results with *Klebsiella-Enterobacter-Serratia* group and tribe *Proteeae* representatives showed but slight qualitative differences. Mixtures of the diluent bacteria also did not cause significant changes in the results obtained. Abbreviations: XLD, xylose lysine desoxycholate; HE, hektoen enteric agar; SS, *Salmonella-Shigella* agar; TSA, Trypticase Soy Agar.

TABLE 3. Isolation of shigellae from artificial mixtures with *Klebsiella-Enterobacter-Serratia* (KES) bacteria^a

Bacterial Mixture	Ratio <i>Shigella</i> to KES bacteria	Final bacterial concn/ml cultured	XLD bacteria		HE bacteria		SS bacteria		TSA bacteria
			Diluent	Test	Diluent	Test	Diluent	Test	
<i>Shigella</i> and KES bacteria	1:10 ²	10 ⁵	>300	81	>300	76	39	10	>300
		10 ⁴	64	13	91	12	7	0	>300
	1:10 ³	10 ⁵	>300	27	>300	21	53	2	>300
		10 ⁴	63	9	82	7	9	0	>300
	1:10 ⁴	10 ⁵	>300	6	>300	7	46	0	>300
		10 ⁴	67	2	74	1	13	0	>300

^a Values represent colonies counted after 18 hr of incubation at 35 C. Results obtained with the two serogroups of *Shigella* and the various members of the KES group approximated the results shown. Colony counts represent the average obtained with all tests performed in duplicate and repeated 7 to 10 times. Results with the several *Escherichia* and tribe *Proteeae* representatives were very similar to those shown. Mixtures of the diluent bacteria did not cause significant changes in results. Abbreviations are contained in Table 2.

were inoculated with a standard loop containing 0.02 ml onto the media used earlier supplemented by the addition of EMB agar. Results obtained (Table 4) bear out the findings obtained with the artificial mixtures. XLD and HE agar permitted the isolation of both shigellae and salmonellae in numbers very close to the theoretical yields of 0 to 1, 4, and 40 salmonellae or shigellae for 100, 1,000, and 10,000 test bacteria added, respectively. Use of SS agar led to excellent isolation of salmonellae but inefficient recovery of shigellae. EMB agar permitted the recovery of both test bacteria, but in fewer numbers than either XLD or HE agar.

These media were employed also in the clinical

TABLE 4. Recovery of salmonellae and shigellae added to feces in stool preservative after 8 hr at room temperature^a

Bacterium	Concn	XLD	HE	SS	EMB
<i>Salmonella typhimurium</i> ..	10 ²	1	2	1	0
	10 ³	6	4	8	2
	10 ⁴	31	30	39	27
<i>S. infantis</i>	10 ²	3	1	2	0
	10 ³	4	3	5	1
	10 ⁴	26	31	37	29
<i>S. newport</i>	10 ²	1	1	3	1
	10 ³	4	5	6	3
	10 ⁴	36	38	35	34
<i>Shigella dysenteriae</i>	10 ²	2	2	0	0
	10 ³	5	6	0	3
	10 ⁴	32	34	2	27
<i>S. sonnei</i>	10 ²	1	1	0	0
	10 ³	4	3	0	1
	10 ⁴	41	43	7	31

^a Abbreviations are contained in Table 2.

laboratory situation. Only stools in stool preservative are accepted and plated, as described before (1, 2), on a variety of selective media as well as inoculated into enrichment broth. The latter is subcultured on certain selective media after overnight incubation. Stool specimens (1,000) were examined using the media under experimental scrutiny among others used routinely. The five test bacteria were isolated with the following frequency: *Salmonella typhimurium*, 23 (three patients); *S. infantis*, 4 (one patient); *S. newport*, 3 (two patients); *Shigella sonnei*, 4 (two patients); *S. dysenteriae*, 2 (one patient). There were, therefore, 36 opportunities for the detection of these organisms directly in the stool as well as after enrichment. All salmonellae and shigellae were isolated from primary plating with HE agar and were recovered as well from all subcultures of enrichment broths. Direct isolation using XLD agar succeeded 28 times; all subcultures from enrichment media were positive. EMB agar permitted direct isolation 23 times, and all salmonellae and shigellae were recovered on subcultures. All but three salmonellae were isolated directly from stool with SS agar, but the shigellae were found only after enrichment.

DISCUSSION

Artificial mixtures of fecal amphibiotic bacteria with salmonellae and shigellae were used to evaluate the recovery of the latter with the use of media designed by Taylor and coworkers (8-10) and King and Metzger (5, 6). Although the selectivity of both media is designed primarily for the isolation of salmonellae, the sensitivity of both far exceeds that of the older, established agar

medium. This is especially true for the recovery of shigellae, organisms neglected primarily because they are not isolated with an efficiency commensurate with their incidence, as Taylor has stated repeatedly. Although both media differ in essential constituents (i.e., HE agar contains salicin and a bile complex, whereas XLD employs xylose, lysine, and sodium desoxycholate), their performance with artificial mixtures was nearly equal. Both failed to suppress the amphibiotic bacteria to the degree which has become accepted for the selective media used in the microbiological analysis of fecal samples. Since this lack of selectivity in no way impairs the sensitivity of either medium, it can be disregarded.

This investigation also stressed again the inability of laboratory studies to mimic conditions in nature (3). The instability of the artificial mixtures leading to a rapid decline of viable test bacteria, especially shigellae, reflects once more the lack of appreciation of the many ecological factors extant under natural conditions. The objectionable instability was overcome by using fecal specimens in a buffered preservative. However, this does not permit the investigator to select companion bacteria and to establish ratios between population components, an absolute requirement for quantitative evaluations.

The differences displayed by the various agars in the laboratory confirm the superiority of the newer ones vis-a-vis the shigellae without risking efficient recovery of the salmonellae. The efficiency of HE agar in recovering both salmonellae and shigellae in direct inoculations is most impressive with the limited numbers studied. The performance of both XLD and HE agar throughout this study was so very close that fur-

ther experience is required to substantiate this performance. The advantages of both media are sufficient to recommend that either, or both, serve in the screening of specimens suspected of harboring salmonellae or shigellae.

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