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Isolation of Shigellae

VI. Performance of Media with Stool Specimens

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The efficiencies of three enrichment broths and four plating media for isolation of enteric pathogens were compared from 1,117 stool specimens. Direct streaking proved to be inferior to enrichment, detecting only 50% of the salmonellae and 61% of the shigellae. By contrast, Selenite Broth (SF) found 90% of the total salmonellae isolates and 82% of the shigellae isolates. Gram-Negative Broth (GN) found 82% and 85%, respectively, but Tetrathionate found only 60% and 39%. Thus, SF and GN were comparable for both salmonellae and shigellae and significantly better than Tetrathionate Broth for both. The plating media compared were MacConkey (MAC), deoxycholate citrate (DC), xylose lysine deoxycholate (XLD), and xylose lysine Brilliant Green (XLBG) Agars. Of the total salmonellae isolated, XLD produced 94%; XLBG, 71%; MAC, 55%; and DC, only 35%. Of shigellae, XLD found 89%; MAC, 75%; XLBG, 63%; and DC, but 27%. The efficacy of XLD is observed to be almost threefold that of DC. The most successful combination of media for the detection of fecal pathogens was GN or SF enrichment broths streaked to XLD plates. These analyses resulted in the isolation of 118 strains of salmonellae and 33 of shigellae.

With the formulation of new enrichment broths and plating media designed to facilitate the detection of shigellae, comparisons between the traditional media for enteric pathogens and these recent additions have been in progress in a locale in which suitable numbers of these organisms exist for valid comparisons. In previous reports in this series, quantitative pure culture studies showed these new media were promising additions to the armamentarium of enteric microbiology (20, 22). Subsequently, pragmatic bench trials with stool specimens have systematically compared the bulk of the traditional media with the new media for efficacy in the detection of all enteric pathogens to accurately delineate the place of the new media in the company of the traditional, and eventually to arrive at the most efficient combination of enrichment broths and plating media for analysis of stool specimens (23, 25, 26). This report is a further step in that direction.

MATERIALS AND METHODS

This study was conducted with routine stools submitted for analysis in southern Louisiana during the period of 6 February to 3 November 1967. Four plating media were streaked directly and inoculated from enrichment broths the following day. The plating media were desoxycholate citrate (DC, BBL), MacConkey's (MAC, BBL), xylose lysine deoxycholate (XLD), and xylose lysine Brilliant Green Agars (XLBG). The latter two were made from XL Agar Base (BBL), with the addition of the necessary supplementary solutions (20). The enrichment broths were Selenite (BBL), Tetrathionate (Difco), and Gram-Negative (GN, Difco). Pathogens isolated were identified biochemically and serologically with group antisera. Many were submitted to the State of Louisiana Public Health Laboratories for identification of serotypes.

RESULTS

The data observed in Table 1 represent 1,117 stool specimens streaked upon 17,872 plates from which 118 strains of salmonellae and 33 shigellae were isolated.

Direct streaking produced 50% of the total salmonellae strains and 61% of the shigellae strains. However, three salmonellae were detected only by direct streaking, so that enrichment accounted for only 115 of the 118 total isolates. Of the positives obtained from enrichment broths, Selenite produced 90% of the salmonellae and 82% of the shigellae. GN broth detected 82% and 85%, respectively, whereas Tetrathionate found only 60 and 39% of them.

The total plate isolations from each of the enrichment broths indicate the relative efficacies of those broths (Table 2).

TABLE 1. *Isolation of enteric pathogens from 1,117 replicate stool specimens*

Organism	Direct				Indirect												Sub-total	Total
					GN Broth				Selenite Broth				Tetrathionate Broth					
	MAC	XLD	DC	XLBG	MAC	XLD	DC	XLBG	MAC	XLD	DC	XLBG	MAC	XLD	DC	XLBG		
Salmonella..	40	52[a]/59	22	43	49	91/94	34	76	53	99/104	34	71	36	63/69	25	40	115	118
Per cent....	(68)	(88)	(37)	(73)	(52)	(97)	(36)	(81)	(51)	(95)	(33)	(68)	(52)	(91)	(36)	(58)	(98)	—
Shigella....	15	18/20	6	14	22	26/28	7	22	22	24/27	7	13	7	10/13	4	6	33	33
Per cent....	(75)	(90)	(30)	(70)	(79)	(93)	(25)	(79)	(82)	(89)	(26)	(48)	(54)	(76)	(31)	(46)	(100)	—
Total	55	70	28	57	71	117	41	98	75	123	41	84	43	73	29	46	148	151
Per cent....	(70)	(89)	(35)	(72)	(58)	(97)	(34)	(80)	(57)	(94)	(31)	(64)	(52)	(89)	(35)	(56)	(98)	—

[a] Number of positives on this medium per total number positive on all four media in this category.

In these analyses, GN and Selenite were of the same magnitude of effectiveness. Selenite produced not quite 3% more salmonella-positive plates than did GN, whereas the latter produced 14% more shigellae than did Selenite. Selenite enrichment resulted in 36% more salmonellae than did Tetrathionate and 39% more than direct streaking. GN enrichment contributed to 65% more shigellae than did tetrathionate and 31% more than direct streaking. Tetrathionate was roughly on a par with direct streaking in efficacy for the detection of enteric pathogens, but for salmonellae slightly superior and for shigellae inferior to about the same degree.

A summary of the isolations by the different plating media is presented in Table 3.

XLD was clearly the superior plating medium for the detection of both enteric pathogens. XLBG was the next best medium for salmonellae, followed by MAC and DC in that order. For shigellae, MAC was second to XLD followed by XLBG and DC. In overall performance, the plates revealed that XLD was the medium of choice, that XLBG and MAC were roughly equal, and that DC performed poorly in this series.

Table 4 gives the actual distribution of shigellae isolated. It can be observed that direct streaking detected most of the *Shigella boydii* strains but only one-half of the *S. flexneri* and *S. sonnei*, whereas, Tetrathionate found all of the *S. flexneri* but only one-third of the other three species. The propensity of the *Enterobacteriaceae* for various strains, as well as the species and genera, to accommodate themselves to certain media in a seemingly capricious way is well illustrated in this table in which the subtotals frequently are at great variance with the actual frequency of isolation on a given medium.

TABLE 2. *Total plates positive for pathogens from enrichment broths*

Organism	Direct streaking	Enrichment broths		
		GN	Selenite	Tetra-thionate
Salmonella.....	157	250	257	164
Shigella.....	53	77	66	27
Total.....	210	327	323	191

TABLE 3. *Total isolations of enteric pathogens on plating media*

Organism	MAC	XLD	DC	XLBG	Total
Salmonella.....	178	305	115	230	326
Per cent.....	(55)	(94)	(35)	(71)	—
Shigella.....	66	78	24	55	88
Per cent.....	(75)	(89)	(27)	(63)	—
Total.....	244	383	139	285	414
Per cent.....	(59)	(93)	(34)	(69)	—

For example, although the direct streaking detected two out of three *S. dysenteriae*, it represents only one of each strain on one plate. In contrast, after GN, all four plating media detected at least two of the three strains, and MAC found all three.

DISCUSSION

There is an inherent lack of correlation between the results noted in pure culture studies and the observations which result from empiric practice. Nowhere in microbiology is that lack

TABLE 4. *Distribution of shigellae isolated from 1,117 stools*

Organism	Direct					Indirect																
						GN Broth					Selenite Broth					Tetrathionate Broth						
	MAC	XLD	DC	XLBG	Subtotal	MAC	XLD	DC	XLBG	Subtotal	MAC	XLD	DC	XLBG	Subtotal	MAC	XLD	DC	XLBG	Subtotal	Subtotal	Total
Shigella dysenteriae	1	1	0	0	2	3	2	2	2	3	1	2	1	2	2	0	1	0	0	1	3	3
S. flexneri	2	2	1	2	2	1	2	1	2	2	3	3	0	1	4	2	3	1	2	4	4	4
S. boydii	5	6	2	4	7	7	8	0	7	8	5	6	0	1	7	2	2	0	2	3	9	9
S. sonnei	7	9	3	8	9	11	14	4	11	15	13	13	6	9	14	3	4	3	2	5	17	17
Total	15	18	6	14	20	22	26	7	22	28	22	24	7	13	27	7	10	4	6	13	33	33
Per cent	(75)	(90)	(30)	(70)	—	(79)	(93)	(25)	(79)	—	(82)	(89)	(26)	(48)	—	(54)	(76)	(31)	(46)	—	—	—

of corroboration anymore apparent than in isolation of enteric pathogens from clinical specimens or food products, animal feeds, and similar substances. This apparent lack of consistency is observed from author to author, or even within the analyses of the same author done on the same premises at different times. When the data on similar types of analyses seem to be diametrically opposed, does it necessarily mean that one author is wrong? These discrepancies and the variations that exist from country-to-country caused one of us to do comparative studies in laboratories abroad to see whether results reported from European laboratories would be reasonably approximated in domestic laboratories. It was observed that at least for the two laboratories in which the author worked, the final analytical results were quite similar despite the use of different media, different methods of analysis, and even different inoculating needles (21, 24).

In the previous paper (26), the poor showing of Selenite Broth is at variance with the more creditable performance observed in these analyses. Since the same technicians and even the manufacturer of media performed the analyses reported in both studies, on the same premises, one surmises that the less-than-constant efficacy of the Selenite, as compared to the GN, may mean that the Selenite performed better in the latter instance, or did the GN merely fare worse? Are the fluctuations merely the observed high and low ends of population curves? Are the percentages of clinically apparent illness, in which the selectivity is more important in isolation than the sensitivity, quite different in the two series, or is it the fact that the preponderance of *S. boydii* (73%) observed previously has given way to *S. sonnei* (52%) in the current study? Certainly, since it is known that Selenite preferentially supports *S. sonnei*, and since the SS Agar, which is most inhibitory to *S. sonnei*, does not appear in this report but did in the previous one (26), one can say that the better showing of Selenite might be explained by the combination of (i) a more amenable group of shigellae for its prowess, and (ii) a more favorable set of plating media for their recovery. Tetrathionate, in contrast, did not live up to its promise for salmonellae but was better than could have been predicted for shigellae.

Ultimately certain broad conclusions emerge in spite of all of these variables and the general inconsistencies between empirical trials. In this series of studies, the effectiveness of XLD and GN as the best combination for both salmonellae and shigellae has been quite apparent. The most sensitive media, MAC and EMB, were shown effective in direct streaking for shigellae; the most inhibitory media, BG, XLBG, SS, and DC, have been more efficacious for salmonellae after enrichment. And both GN and Silliker's Broths have produced better results with the plating media than have the traditional Tetrathionate and Selenite.

Many of the variables at work in these analyses are not subject to our control because the specimen is an unknown with all that that portends. However, the failure to *control* variables is not to be construed as failure to *understand* variables. Toward this end, the discussion of some particular sources of variation is offered in hopes that the apparent inconsistencies observed in studies of this nature will be better understood.

Factors causing variations in plating media. Jameson (7) provided great insight into the dynamics of enrichment. His lucid detailed discussion of the complex relationships that exist in mixed cultures and the effect that they exert on lag time, generation time, and molar con-

centrations explains many of the variations which are more frequently observed than understood in these comparative studies of media efficacies. Both he and McCoy (11) reported that plating media poured at the same time but used on different days frequently produced major variations in efficacy for detection of pathogens. The loss of inhibition by Bismuth Sulfite Agar (Difco), after periods of refrigeration up to 96 hr before use, was illustrated by the latter author. Obviously, then, one sees that variations due to different manufacturers' brands of prepared media in which different peptones and different bile salts or dyes are used may be great sources of dissimilarities in enteric results. Even the variations from one lot number to another occasionally are so noticeable that one discards or demands replacement of an individual bottle of medium after using one or two portions. Since all enteric media attempt to reach an effective compromise between sensitivity for fastidious pathogens and selectivity against the ubiquitous coliforms, exceedingly minute chemical changes wreak havoc on the delicate sensitivity-selectivity balance of these media, whether it be broth or plate. This balance is so fragile that the cumulative effect of the inhibitors in a loopful of enrichment broth actually influences the plating medium. Georgala and Boothroyd (3) demonstrated that when Selenite-F (Leifson formula) was used as a diluent for salmonellae cultures, marked additive toxic effects were evident on six different plating media, as compared to salmonellae suspended in 0.1% peptone water. In the present study (Table 1), XLBG performed very creditably for isolation of both pathogens after GN enrichment but poorly after Selenite and Tetrathionate, especially for shigellae. When any of these plating media is allowed to dehydrate, the eH is changed, the concentration of the inhibitor is increased, and the most visible effect is sparse growth or dwarf colonies. In the clinical laboratory where it is often practical to make up several weeks' supply of media at a time, minimal change results if the freshly poured plates are put back into the mylar bags that the sterile plastic plates come in and are hermetically sealed by folding over and taping the top of the bag. A 2-week storage period of unsealed plates in the low-humidity environment of the refrigerator is very deleterious to almost all of the enteric media, and aberrant results will occur.

Factors causing variations in enrichment broths. In the behavior of what purports to be a simple, well-known enrichment medium, Selenite Broth, with which we have had experience spanning a third of a century, one finds that knowledge of its mechanism of inhibition and factors affecting it are far from complete. Leifson (8) discussed at length many of the substances which influenced the toxicity of Selenite Broth, such as *p*H, inorganic salts, particularly phosphates, beef extract, meat infusion, casein and other proteins, and even bacteria themselves. He emphasized the necessity for balancing the amount of inhibitor, not only against its own neutralizing ingredients (i.e., peptone, phosphates) but also against those of the anticipated specimen, for which he made specific formulas, such as for milk (Selenite-M), sewage (Selenite-S), and feces (Selenite-F). Leifson and Jameson (7) both revealed, in pure and mixed cultures and in the presence of feces, that what constitutes optimal concentrations of Selenite for the majority of salmonellae was highly inhibitory for serotypes such as *Salmonella choleraesuis* and *S. gallinarum* and for *Shigella flexneri*.

Vassiliadis (28) found that 105 shigellae were isolated on DC agar (Hynes modification, Oxoid) directly, but only 16 of these were recovered after Selenite (Oxoid) enrichment of which 13 of 25 were *S. sonnei*, and only 3 of 78 were *S. flexneri*. North and Bartram (13) observed that two commercially prepared Selenite Broths were not comparable in their ability to recover salmonellae but that the addition of cystine and careful adjustment of the phosphate content brought the unsatisfactory medium up to par with the other. The peptones used were mainly responsible for these differences. Rappaport (14) also found that only certain peptones produced satisfactory results in his enrichment broth. In studies using a fastidious *Salmonella paratyphi*, tryptone alone produced good growth without requiring the addition of glutamine. Smith (18) reported that the selective action of Selenite was not dependent upon reduction of sodium Selenite, but that interference with the sulfur metabolism was the probable mode of action. Addition of cystine and pentathionate reduced the toxicity of Selenite for certain organisms. The action of the peptone seems to be one of Selenite binding by its peptides. The growth-stimulating effect of a fermentable carbohydrate was also demonstrated, which mitigates in favor of the use of a carbohydrate other than lactose for detection of pathogens. Weiss et al. (29) demonstrated that the kinetic aspects of Selenite uptake were the factors determining susceptibility with *Escherichia coli* incorporating twice as much in its cells as resistant *Salmonella thompson* and *Proteus vulgaris* strains. Silliker (15) reported that Selenite-F, designed specifically for the addition

of feces, is in fact more efficient for the quantification of salmonellae in foods if 10% autoclaved feces are added to it before the food sample.

Food microbiologists, accustomed to enumerating salmonellae, know of the problems of the deleterious effect of soluble foods on the inhibitors in enrichment broths. Silliker and Taylor (17) found that highly soluble proteins, such as albumin, nullified the inhibitory action of both Tetrathionate and Selenite broths when diluted even as much as 1,000-fold. Recognition of this phenomenon in food specimens led North (12) to recommend the practice of inoculating noninhibitory broths first (preenrichment) and then to inoculate Selenite or Tetrathionate Broths subsequently with only a loopful of the 18-hr broth.

In the clinical laboratory, there are no comparable studies on the effect on inhibitors caused by quantitative variations in specimen size, i.e., a bean-sized lump of feces added to 5 ml of Selenite, as compared to a swab wiped on the inside of the tube, or qualitative variations, e.g., blood, mucus, or liquid stool specimens instead of formed feces. Subsequently, the clinical microbiologist seems to be much less aware of the possibility that quite different results might be obtained under the different circumstances. Rappaport (14) states that a 1:1,000 dilution of feces produces superior results with his broth than undiluted feces; however, he fails to relate how he secures that dilution. One must be constantly reminded that microbiology is a *technique science* and that the failure of most authors to reveal their detailed methods is as much caused by their attitude that those details are unimportant as by the editor's insistence that they be omitted in deference to the current astronomical printing costs. These essential points of variance in technique are not apparent, therefore, in many publications.

Effect of microbiological numbers on variation. The detection of pathogens is influenced by the numbers in two basically different ways. First of all, there must be sufficient *absolute* numbers of the pathogen in the specimen to initiate growth in the receiving medium. Second, the *ratio* between the relative numbers of the pathogen and its competitor must not be so disparate that a plate streaked with the mixed flora is overgrown by the competitor so as to conceal the pathogen.

The role of absolute numbers may be visualized as being responsible for the findings with sewage polluted waters reported by McCoy (11). He observed that with lightly contaminated water, the greater the total volume, the higher the percentage of salmonellae positives; e.g., 450-ml portions produced 33% positives but 2,700-ml samples yielded 71% positives. He poured his samples through a sterile cotton plugged filter, then immersed the plug in enrichment broth, for a simple but obviously effective concentration method. He also correlated the numbers of salmonellae in the sewage to the time that it took to obtain all possible positives by subculturing on each of 4 successive days. When there were only one or two salmonellae per 100 ml of water, as determined by the most probable numbers method, he obtained a significant number of positives on each of the 4 days. From 3 to 9 per 100 ml, 24- and 48-hr (rarely, 72-hr) subcultures were sufficient to find all positives, and, when the salmonellae count reached 12 per 100 ml, a single plating at 24 hr was adequate. Time thus became a measure of the inoculum density because the molar concentration of the pathogen necessary to detect it in a mixed flora was reached more quickly as the size of the inoculum of salmonellae increased.

Preenrichment (12, 19, 27) was of value over traditional enrichment broths when the numbers of salmonellae were exceedingly small. The absolute number necessary to initiate growth in a noninhibitory broth was much lower than for the inhibitory broths, and the debilitated physiological condition of the pathogens found in processed food products widened the gap even further.

McCall et al. (10) did quantitative studies on stools and rectal swabs following an institutional outbreak of *Salmonella derby*; they found that rectal swabs were inferior to fecal specimens when the numbers of salmonellae decreased to less than 1,000 per gram. Short-term carriers (less than 4 months) had salmonellae counts from 720 to 61,000 per gram; but on two occasions, two consecutive negative stools were obtained from the six patients. Long-term carriers (1 year or more) had counts of 10 to 6,900 per gram. The eight long-term carriers had varying periods of consecutive negative stools up to as many as nine, in one case. The short-term carriers excreted greater numbers of salmonellae. The small inoculum afforded by the rectal swab is probably too low in absolute numbers of pathogens when stool counts are less than 1,000 per gram. Similarly, the long-term carrier has both low counts of pathogens and large daily fluctuations in counts. Again, failure to detect is probably a variable of absolute numbers, but with overtones of the ratio factor, because, unlike dilute sewage in which the low-pathogen

count presumably also has a corresponding low-coliform density, the formed stool will not vary greatly in its coliform count whether the enteric pathogen is heavily or lightly seeded.

As the numbers of pathogens increase to the point where a growth-initiating level is guaranteed in the specimen, the relationship is optimal between pathogen and medium. The interference by the competitor organisms in the specimen changes that relationship, however. The relative numbers of both or the ratio between coliforms and pathogens then becomes the determining factor in success. Silliker (16) found that traditional inhibitory enrichment broths produced superior results over preenrichment when the absolute numbers were high for both organisms or when the coliform population was much greater than that of the pathogen. Lendon and MacKenzie (9) diluted sewer swabs or bone meal to 1:10 to 1:1,000 dilutions for maximal positives, as did Rappaport with feces at 1:1,000 dilutions (14). In contrast, Collard and Unwin (2) and Iveson and Kovacs (6) used Rappaport's medium to compare the diluted with undiluted feces and found more salmonellae in the latter. Therefore, one cannot say with certainty that a 1:1,000 dilution of feces will produce more salmonellae than the undiluted, because from specimen-to-specimen, both absolute numbers of pathogens and the ratio of pathogen to competitor are unknown covariables. In the high-count diarrheal stool of the well-developed clinical case, the diluted stool should offer an excellent optional method for increasing positive isolations. The chronic carrier or the subclinical case with low-count pathogens in formed feces, conversely, requires that the undiluted stool be inoculated into the most efficient selective broth available for successful detection.

In essence, then *sensitivity* correlates with small absolute numbers and is the primary factor in preenrichment as a successful method for low-count foods, water, or sewage samples. *Selectivity* becomes the major factor (i) when both the pathogen and its competitors occur as high absolute numbers, or (ii) when the disparity between the relative numbers of coliforms and pathogens constitutes a ratio greatly to the disadvantage of the latter.

Determination of statistical significance of variables. The foregoing discussion has been concerned with biological factors influencing the relationship between organisms and their recovery media. The resultant variations are frequently great enough so that gross inspection of the data constitutes a valid analysis of significance and appropriate conclusions may be drawn with confidence. The rules of population sampling and the significance of variations, however, must be accorded their importance in this type of investigation, and failure to appreciate this fact has led to some erroneous conclusions. Cochran and Cox (1) chose (most aptly for our purposes) illustrations using two media for isolation of salmonellae or multiple media comparisons to introduce a simple formula more valid for this kind of analysis than the usual fourfold table of X^2. They compare only the trials in which isolation occurred on one medium but not the other, and they discard those results either positive or negative on both. Thus, if a is the number of $(+-)$ blocks and b represents $(-+)$ blocks, then the formula is $X^2 = (a - b - 1)^2/(a + b)$. The simplicity of the formula makes it possible to do a rapid test of significance on data reported by other authors who did not analyze their figures; frequently one finds that they placed unwarranted confidence in observed differences which are not statistically significant.

A second source of statistical error is that one may not legitimately compare results obtained on one medium with the total aggregate from two or more media, since one medium thus represents only one-half or one-third of the sum total of inoculations. Thus the assumption, for example, that the ability of DC or MAC to find some strains of salmonellae or shigellae not found on XLD is justification for multiplicity of media could only be proved by having inoculated a number of XLD equal to the total of the aggregate and by comparing positive isolations only on that basis. Guinée et al. (5), with the aid of a statistician and with carefully homogenized feces and pig lymph nodes, analyzed their salmonellae isolations obtained with Selenite and Tetrathionate Broths and drew conclusions at variance with those drawn from their previous study (4).

The third source of error is the axiom that one medium will fail to find as many enteric pathogens as two, or three, or four, and this has been stated in print innumerable times. *That is not true!* Statistically, it will be true (i) only when all of the media are approximately of the same order of efficiency, or (ii) the sampling error is large enough to cause random successes merely because one medium gets a sufficient inoculum of pathogen and another does not. If, however, one medium is greatly superior in its sensitivity and selectivity to the others, it alone will outperform the aggregate. In this series of studies, XLD detected many more salmonellae and shigellae than the aggregate of E M B, SS, and BG Agars combined (25), or the combination of MAC and DC

(Table 3). One may hopefully conclude, then, that as more efficient media are produced for enteric work in the clinical microbiology laboratory, the distressing proliferation of media and manipulations of the specimen will abate concomitantly with greater ease and accuracy of analysis.

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