Effect of Temperature on Transport and Plating Media for Enteric Pathogens

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The effect of wide variations in incubation temperatures and long periods of incubation on transport and enrichment broths and plating media was determined by exhaustive analysis of 132 diarrheal stools for salmonellae and shigellae. Homogenized stools were streaked onto eosin methylene blue (EMB), *Salmonella-Shigella* (SS), and xylose lysine deoxycholate (XLD) agar plates, and into saline, Cary-Blair (CB) transport medium, and Selenite F and gram-negative (GN) enrichment broths. Incubation temperatures were compared at 20 C, 35 C, 40 C and ambient, and over a range of 4 to 52 C for media incubated in an insulated picnic cooler in an auto trunk. At 1, 2, 4, and 7 days the plates were observed, and the broths were subcultured. Each stool was streaked to 12 plates for 48 observations and pickings, and to 48 tubes, subcultured to 192 plates, for a total of 240 observations for pathogens. Analysis of data from 6,246 *Salmonella*-positive plates showed direct streaking to be most effective after 2 days of incubation, but broths were equally effective at 1 or 2 days. By day 4 many plates were overgrown, and both plates and broths showed diminution of positives by about 10% and at day 7, 19%. The 2,434 *Shigella*-positive plates were more demanding in all times and temperatures of incubation than salmonellae. Although at day 2 best results were obtained on direct streaking, shigellae die-offs in broths were excessive, with positives declining 23.7% by day 2, 49% by day 4, and 60% by day 7. Direct plating of both pathogens was poor at 20 C with about 48% success, but salmonellae preferred higher temperatures (35 and 40 C), whereas shigellae chose 35 C and ambient, which averaged 28 C for the 10-month study. Temperature was immaterial to salmonellae in broths with ambient slightly better than 35 C, but shigellae preferred 20 C and showed a 50% failure rate at 40 C, ambient being equal to 35 C. The preferential rank of broths in efficacy was GN > selenite > saline > CB > direct for salmonellae; for shigellae, GN > saline > direct > CB > selenite, with selenite proving to be unsuitable for shigellae. Plating media preferences were XLD > EMB > SS. Ten of 39 shigellae strains could not be recovered from the selenite and SS media combination, the many replications notwithstanding. The effectiveness of salmonellae and shigellae detection at ambient temperatures in Louisiana during the 10-month study period, as compared to controlled incubation temperatures, indicates that satisfactory enteric bacteriology can be done in warm climates without constant temperature incubators.

A tacit assumption by microbiologists that the optimum temperature for growth of enteric pathogens equates with the obligatory temperature for growth probably exists simply because laboratories have always had incubators and they have always been used at the optimum temperature for the growth of human and animal pathogens. In retrospect there must be cognizance that the normal life cycles of many human pathogens include survival outside of

the human body. Salmonellae, in particular, may survive all of the rigors of food processing, including heating, drying, treatment with chemical agents, chilling, freezing, and long periods of storage at ambient temperatures, and yet remain infective when ingested by man or animals (10). Their ability to survive under the most adverse of conditions is undoubtedly a major factor in their ubiquity and their involvement in an estimated two million clinically apparent salmonellosis cases per year in the United States, where, presumably, the water is

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safe to drink. If these pathogens are able to survive not only under a wide range of nonoptimal temperatures, but even under deleterious conditions, then it is likely that the restriction of temperature to 35 C for detection of these organisms may not be mandatory, and that there is a wider range of temperatures which may not necessarily be best but may still be satisfactory for stool analyses for these organisms.

In the previous report in this series of studies (9), transport and enrichment broths and primary plating media at rigidly maintained incubator temperatures, optimal and otherwise, were observed for efficacy in detection of stool pathogens. It was concluded that the temperature ranges examined, 20 C, 25 C and 35 C, at 1-day and 2-day incubation times, were less important than the choice of media for the analyses. In the study here presented, the least efficient broth media have been abandoned but wider parameters of incubation temperatures, including ambient temperatures for southern Louisiana, and longer periods of incubation have been examined.

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MATERIALS AND METHODS

Diarrheal stools received by East Jefferson General Hospital, Metairie, La., during the period 23 August 1972 to 15 June 1973 were analyzed using four transport or enrichment broths, three plating media, four incubation temperatures, and four incubation time periods.

Because of the great number of replications involved in this study, and since negative results are unrewarding, freshly collected diarrheal stools from inpatients and outpatients, and occasionally some from other hospitals, were analyzed by routine methods for salmonellae and shigellae, and the stools were saved and refrigerated. If the stool proved to be positive, then it was subjected to the experimental protocol described herein. In many cases, fresh stools were collected from patients already known to have an enteric pathogen, so that stools utilized ranged from freshly passed to those refrigerated up to 3 days. During the periods of infrequent stool positives, some diarrheal stools were employed in these studies without prior screening and these account for the negatives in this report.

An emulsion of stool solids, liquid, mucus, and blood was made from the patient's specimen. Approximately 3 ml of this was suspended in 15 ml of sterile saline and thoroughly mixed on a Vortex mixer. Four tubes each of saline, Cary-Blair (CB) (2) transport medium, and Selenite F and gram-negative (GN) enrichment broths received 2 ml each of the stool suspension. A sterile swab dipped into the remaining suspension was used to inoculate four

plates each of eosin methylene blue (EMB), *Salmonella-Shigella* (SS), and xylose lysine deoxycholate (XLD) agars. One set of each of the broths and plates was incubated in 20 C, 35 C and 40 C incubators. The fourth set, at ambient temperature, was placed in a styrofoam picnic cooler in the trunk of the car of one of us. The insulating properties of the styrofoam cooler were used to diminish the temperature extremes which would result from the metal skin of the car parked in the sun. Temperatures in the container were recorded by a clock-driven circular chart, 7-day recording thermometer, -30 F to 120 F [ca. -34 to 49 C] range (Bacharach Instrument Co., Pittsburgh, Pa.). The plates initially inoculated (direct) were read, and suspect colonies were picked after 1, 2, 4, and 7 days of incubation at each temperature. These plates were returned to their various temperatures after each observation. The direct method used only 12 plates, but with four observations each, for 48 examinations for pathogens per specimen.

In the indirect method, the four broth tubes, incubated at four different temperatures, were each streaked onto EMB, SS, and XLD plates, respectively, for 48 such platings. The tubes were returned to their respective incubation temperatures, but the plates were incubated uniformly at 35 C and observed for pathogens only at 24 h, so that only the broths were subjected to temperature variance, not the subcultured plates. By contrast, in the direct method, the plates themselves were incubated at the various temperatures. Each tube was subcultured after 1, 2, 4, and 7 days of incubation for a total of 192 plates and observations for the presence of enteric pathogens. The combination of direct and indirect methods produced 204 plates and 240 observations per stool specimen. The analysis of 132 stools resulted in the inoculation of 26,928 plates and 31,680 recorded observations. Of these, 6,246 plates were positive for salmonellae and 2,434 for shigellae.

Suspect-pathogen colonies were picked in duplicate, or more, to dulcitol lactose sucrose iron (BBL) (7) agar slants, lysine iron agar slants, or r/b tubes (Diagnostic Research, Inc., Long Island, N.Y.), usually in a combination of two of the three media. Isolates resembling *Salmonella* or *Shigella* biochemically were typed serologically to group designations, and many of the salmonellae were sent to the Public Health Laboratories of the State of Louisiana in New Orleans for further identification of serotypes.

RESULTS

The distribution of salmonellae isolated after various periods of incubation on plates streaked directly, and in transport or enrichment media, may be seen in Table 1. Plates streaked directly yielded significantly better results after 2 days of incubation than after 1, 4, or 7 days ($P < 0.01$). By day 4, plates were frequently overgrown to the point that colonies of pathogens were no longer recognizable. The day-2 isolations represented a 32% increase over 1 day, but

the day-4 and -7 isolations were decreased by 10.0 and 21.6%, respectively. By contrast, the efficacies of the liquid media were diminished less at 1 or 2 days, but by day 4 declining numbers of recoverable salmonellae resulted from all four media. Saline positives decreased by 11% from day 2 to day 4 and CB decreased by 10.4%, but selenite decreased only by 3.6% and GN by 9.3%. By day 7 the failure to recover salmonellae was even more marked. CB, which was least effective in isolations on day 1, showed a 24.3% decrease, almost one-fourth of its positives by day 7. GN, the best broth, dropped 18.8% and saline decreased 19.4% but selenite, which was comparable to saline on the first 2 days, was superior to it at day 4 and 7, decreasing only 14%. The preferential order for the broths then is GN first, with greater recoveries than saline or selenite and still best after 7 days, followed by selenite, least affected by the 7-day holding period, then saline and the least efficacious, CB. Thus the inhibitory broths proved to be better for salmonellae than the noninhibitory broths when long periods and wide parameters of incubation temperatures occurred.

The distribution of shigellae isolated is seen in Table 2. The recovery rate of shigellae is poor by comparison with salmonellae under the same conditions. For example, the die-off of shigellae by the second day in the broths (23.7%) exceeded the failure rate in salmonellae

on day 7 (18.8%). By day 4 shigellae recoveries had decreased by 49.4%, and by day 7 positives by all methods had decreased by 59.7%. In preferential rank of efficacy, GN and saline were superior to direct stool plating for shigellae, with CB less effective than direct, and selenite so poor as to be unacceptable. Direct streaking of shigellae, as in the case of the salmonellae, produced the maximum number of positives after 2 days of incubation, there being 22.3% and 24.3% fewer positives, respectively, of the two pathogens recovered after only 1 day of incubation. The major factors here were (i) the effect of the low temperature, 20 C, which resulted in pin-point colonies with no identifying features on EMB and XLD at 24 h of incubation, and (ii) the distinct preference of SS agar for higher temperatures, as it usually showed no growth of either pathogen at 24 h at 20 C or ambient temperatures.

The recorded ambient temperatures which occurred in the 10-month duration of this study are shown in Table 3. The temperature extremes from the February low of 4 C to the September high of 52 C certainly afforded the specimens a sufficient temperature range to exert any untoward effects on the viability of the pathogens that one could reasonably expect. The averages of the highs and lows produced a 20 C differential, greater than the difference between the 20 C and 35 C temperatures of the incubator. For 6 months the means

TABLE 1. *Distribution of 6,246 Salmonella-positive plates*

Day	Direct	Saline	CB	Selenite	GN	Indirect total
1	203 (24.3) ^a	354 (0.6)	338	358	388 (0.3)	1,438
2	268	356	324 (4.1)	352 (1.7)	389	1,421 (1.2)
4	241 (10.1)	317 (11.0)	289 (14.5)	339 (5.3)	353 (9.3)	1,298 (9.7)
7	210 (21.6)	287 (19.4)	256 (24.3)	308 (14.0)	316 (18.8)	1,167 (18.8)
Total	922	1,314 (9.1) ^b	1,207 (16.5)	1,357 (5.5)	1,446	5,324

^a Number of positive plates from each analytical method; numbers in parentheses indicate the percentage of decrease in positives.

^b Numbers in parentheses indicate the percentage of decrease in positives compared to GN total.

TABLE 2. *Distribution of 2,434 Shigella-positive plates*

Day	Direct	Saline	CB	Selenite	GN	Indirect total
1	160 (22.3) ^a	223	177	77	227	704
2	206	175 (21.5)	131 (26.0)	49 (36.4)	182 (19.8)	537 (23.7)
4	130 (36.9)	118 (47.1)	89 (49.7)	35 (54.5)	114 (49.8)	356 (49.4)
7	57 (72.3)	81 (63.7)	72 (59.3)	32 (58.4)	99 (56.4)	284 (59.7)
Total	553	597 (4.0) ^b	469 (24.6)	193 (69.0)	622	1,881

^a Number of positive plates from each analytical method; numbers in parentheses indicate the percentage of decrease in positives.

^b Numbers in parentheses indicate the percentage of decrease in positives compared to GN total.

were 20 C to 26 C, and for 4 months the means were 32 C to 36 C. It was not readily discernible that there was any difference in the data in the colder months than in the warmer, however, because in the colder months there were fewer diarrheal disease patients and the negative stools examined came almost entirely during those winter months. It appears that, when the season for diarrheal diseases exists, the ambient temperatures are also the best for the laboratory detection of those pathogens.

The greatest number of salmonellae isolations occurred in April (eight cases) and May and August (nine each); the fewest occurred in December through February (only two). Shigellae were most numerous in April (ten) and May (nine), but a rather mild February produced six cases also. The negative stools occurred most frequently in September and October but the only *Arizona* was detected in October.

The effect of temperature on the distribution of positive plates is shown in Table 4. Neither

pathogen grew well on direct plates at 20 C; salmonellae isolations decreased by 46% and shigellae by 50% by comparison with 35 C. However, in the indirect method, shigellae again (9) showed a preference for temperatures lower than 35 C, with 20 C producing the maximum isolates and ambient being equivalent to 35 C in efficacy. By contrast, at 40 C 50% of shigellae were missed. Salmonellae in the direct showed little preference between 35 C and 40 C (-1.1%) but were less tolerant of ambient temperature (18.2%), but indirect preferred ambient by a slight margin over both 20 C (2.3%) and 35 C (0.8%). Even 40 C showed only an 8.5% decrease in positives, indicating the flexibility of the broths in comparison with direct plating methods for salmonellae isolations. By contrast, at 40 C one would isolate only 50% of the shigellae found at 20 C. By both direct and indirect methods, and for both pathogens, ambient temperatures proved acceptable as the lower temperature was preferred to 40 C in three of four categories.

In Table 5 the performance of plating media is shown. The superiority of XLD for both pathogens upon either direct or indirect inoculation is obvious, with recoveries from 90.0 to 97.9%. Shigellae preferred EMB to SS by more than two to one, but EMB still found but 63 to 65% of the total. Salmonellae showed a slight preference for SS over EMB (68.9 versus 59%) on direct plating but exhibited a reversal on indirect with EMB (71.9%) over SS (63.7%).

Selenite broth and SS agar proved to be excessively inhibitory to the shigellae. Nineteen of 39 strains failed to grow on any of the 78 replicate SS plates per specimen, and 20 were not recovered from any of the 48 selenite broth subcultures. Five of the six *S. flexneri* failed to grow on SS, four of six failed to grow in selenite, none of the five *S. dysenteriae* were detected from the selenite-SS combination, and only one grew on any SS plate. For that matter, only 10/39 shigellae strains could be recovered

TABLE 3. Ambient temperatures of incubation

Month	Recorded temp (C)		
	Low ^a	High	Mean
September 1972	25	52	36
October	21	41	35
November	9	31	24
December	10	30	21
January 1973	12	30	24
February	4	30	20
March	24	28	26
April	16	30	25
May	24	48	32
June	23	47	35
Avg	17	37	28

^a The low and high recorded were the temperature extremes for the month, whereas the mean is the average of the weekly temperature records for that month and therefore may not be the midpoint between stated highs and lows.

TABLE 4. Distribution of 8,680 positive plates by temperature

Incubation temp (C)	Salmonellae		Shigellae	
	Direct	Indirect	Direct	Indirect
20	149 (46.0) ^a	1,339 (2.3)	95 (50.0)	604
35	276	1,360 (0.8)	190	491 (18.7)
40	273 (1.1)	1,254 (8.5)	127 (33.2)	302 (50.0)
Ambient	224 (18.2)	1,371	141 (25.8)	484 (19.9)
Total	922	5,324	553	1,881

^a Number of positive plates from each analytical method; numbers in parentheses indicate percentage of decrease in positives.

TABLE 5. *Performance of plating media*

Method	EMB	SS	XLD	Total ^a
Salmonella				
Direct	245 (59.0) ^b	286 (68.9)	391 (94.2)	415
Indirect	1,640 (71.9)	1,452 (63.7)	2,232 (97.9)	2,279
Shigella				
Direct	195 (63.3)	78 (25.3)	280 (90.9)	308
Indirect	674 (65.1)	265 (25.6)	942 (90.9)	1,036
Total	2,754 (68.2)	2,081 (51.5)	3,845 (95.2)	4,038

^a Total number of positives from any combination of the three plates.

^b Number of positive plates resulting from each analytical procedure; numbers in parentheses indicate the percentage of total.

from selenite-SS analysis even with 16 replications per stool. One must conclude that this is a highly unsatisfactory combination of media, although these media are still widely used for this purpose in a great many clinical microbiology laboratories.

The 132 stools resulted in isolation of one *Arizona hinshawii*, 39 *Shigellae* sp., 47 *Salmonella* sp., and 45 that were negative for these pathogens. The salmonellae were identified as: *S. agona* (two), *S. anatum* (two), one each of *S. give*, *S. infantis*, *S. javiana*, and *S. mississippi*, *S. st. paul* (five), *S. thompson* (four), and *S. typhimurium* (nine). The remaining salmonellae were typed by group only: B (eight), C1 (nine), and one each of E1, E2, G, and I. The shigellae were: *S. dysenteriae* (five), *S. flexneri* (six), *S. boydii* (two), and *S. sonnei* (twenty-six).

DISCUSSION

The purpose of this study is to delineate the parameters of efficacy of times and temperatures for detection of salmonellae and shigellae in diarrheal stools. If those parameters are wide enough to encompass the ambient temperature range for the tropics, neotropical, and warm areas of the temperate zones, then the efficacy of the microbiology laboratory will be extended, since specimens can be held for longer periods of time and through a greater temperature range with certainty that the recovery of enteric pathogens will be within permissible limits when analyzed at the laboratory subsequently. The corollary is that an expeditious choice of media may make it possible to detect stool pathogens without direct recourse to the laboratory. If, for example, we compare the most effective combination of media, GN streaked to XLD plates, at 35 C and at ambient temperatures, the ambient temperature produced more isolates than 35 C, 159 versus 157

salmonellae and 90 versus 86 shigellae. With performance like that it would seem to be prejudicial to even refer to ambient temperature incubation as disadvantaged. But in direct streaking, ambient temperature is at a disadvantage because of colony size at 24 h. The direct method of salmonellae on XLD plates at 35 C and ambient temperature produced 118 and 97 positive plates, respectively, over the 7-day incubation period. Although these differences were not significant ($P > 0.1$) if the first day were to be omitted, during which the ambient plates did not produce identifiable colonies, the data for days 2 through 7 were 85 positives at 35 C to 80 at ambient temperature, a truly insignificant difference. Thus ambient temperature is shown to be equal in accuracy of analysis, but 1 day slower in time for analysis for the salmonellae. In fact, as day 7 approaches, ambient temperature retains its usefulness better because plates at 35 C become unreadable because of overgrowth of coliforms and protei, etc. Ambient is not satisfactory for shigellae by direct streaking, however; 35 C is superior by a 98 to 70 margin ($P < 0.05$).

The shigellae in general pose problems not found in salmonellae detection. Their failure to survive in mixed flora has been documented by Hentges (4) and Isenberg et al. (5) as well as by ourselves. The die-off of shigellae in all of the broths at any period other than 1-day incubations makes it necessary to redefine acceptable. Is the 20% decrease in positives seen in GN and saline in Table 2 acceptable? Certainly the 36% decrease seen with selenite is of dubious acceptability. But then selenite is not an acceptable medium for shigellae under the best of circumstances, with only 77 positives versus 227 for GN and 223 for saline at 24-h incubation. By day 4 the positives from all broths have decreased by 49% and at seven days by 60%. With the combination of GN and XLD the shigellae

recoveries on day 2 are comparable with day 1 at both 35 C and ambient temperature, 56 versus 61, respectively, and therefore acceptable. After day 2, the analytic accuracy decreases, with 7-day incubation producing only 24/61 positives (60.7% loss) even with the most favored media combination. The need for a better holding-transport medium for shigellae still exists (3, 7).

Salmonellae are obviously robust organisms quite competent to survive in their ecological society and to be detectable under almost any circumstances. CB medium and direct streaking showed losses of 24 and 22%, respectively, by day 7 of incubation (Table 1). Considering that these figures include the worst temperatures and worst media combinations, acceptable becomes an all-encompassing definition. The observation of only a 14 to 18.8% decrease in isolations for GN and selenite for 7-day incubation, and with ambient temperature fluctuations of 4 C to 52 C, indicates the feasibility of using these media as transport-enrichment broths under field conditions when laboratory facilities are not immediately available. The projected recovery of 84% positives on the average is an acceptable degree of efficacy especially since, were the incubation periods to be less than 7 days and the temperatures less than the extremes recorded here, even higher recovery rates would accrue. Of course, missing salmonellae when there are 192 plates resulting from a single stool is not as easy as when only three plates are streaked. Actually, however, there are relatively few times in these analyses that GN broth, XLD plates, and 24 h at either ambient temperature or 35 C did not find the pathogen, so that the 192 replications were not that much more effective than three plates each by direct and indirect methods. In Table 5, the theoretical totals, any positive found by any medium or any method, compared to the XLD-detected positives showed XLD to be 91% as effective as all combinations for shigellae, and 94 and 98% for direct and indirect detection of salmonellae, respectively.

Our experiences with the media and time-temperature relationships under consideration lead us to conclude that if we were to have to do stool analyses under field conditions, that is, carrying what we could in a vehicle from village to village, whether it be in the tropics or any warm area of the globe, and with no opportunity to reach an established laboratory within a week, our procedure would be as follows. We would carry tubes of sterile saline and GN broths, cotton-tipped swabs, sterile or non-sterile, and XLD plates. We would inoculate one tube each of saline and GN broth from the

stool. At 24 h we would streak an XLD plate from each, saving the broths. At 24 and 48 h we would examine and pick the plates, the 48-h plate being used only if the 24-h plate were negative, but negatives would be retained and inspected daily until overgrown. As for identification, the two-tube systems, r/b 1 and 2, or dulcitol lactose sucrose iron medium (8) and modified lysine broth (1) would suffice for preliminary biochemical characterization, and the slants would be adequate for maintenance of the isolates until serotyping could be performed at the laboratory. This greatly simplified protocol, lacking in sophisticated instrumentation, should produce acceptable results for surveying the status and identity of diarrheal disease in geographical areas deficient in laboratory facilities. Since transport by rough-terrain vehicles, light aircraft, or helicopters is not uncommon to even relatively remote areas, laboratory teams can move into and out of such areas more easily than they can find facilities competent to do enteric analyses. It is hoped that the study herein presented will prove of value in pragmatic clinical microbiology.

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