Laboratory and Clinical Investigation of Recovery of *Salmonella typhi* from Blood

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An experimental study was undertaken of isolation of *Salmonella typhi* from artificially infected whole blood and blood clot prepared either with small numbers of freshly isolated strains or with the *S. typhi* Ty2 strain. Results showed that 8 ml of whole blood required a minimum of 50 ml of bile salt broth to dilute the bactericidal effect of the serum. However, the isolation rate for recovery in 210 cases of enteric fever was only 64% when 50 ml of medium was used, as compared to 92% recovery from blood clot from the same group incubated in streptokinase bile salt broth. This probably reflects a very low grade bacteremia in many of the patients. Further investigation showed that, ideally, between 150 and 200 ml of medium is required for satisfactory whole-blood culture. Both the experimental study of artificially infected clots and recovery rates from patients indicate that rapid clot dissolution with streptokinase is preferable to whole-blood culture; this experience has been amply confirmed by almost 5,000 cases over a 20-year period. A 15-ml volume of bile salt broth with 100 U of streptokinase is adequate for the clot from 8 ml of blood, and savings on media and incubator space have considerable cost benefits in developing countries.

Isolation of *Salmonella typhi* from blood is the diagnostic method of choice in typhoid fever. However, the reported incidence of positive isolates varies enormously. In a series in Delhi, India, only 527 isolates were obtained from 5,735 suspected cases (9.2%) (5). This compares with 175 isolates in 243 cases (72%) in Rhodesia (8) and 219 isolates from 298 clinical cases in a pediatric group in Natal (73%) (4). Our own experience, also in Natal, gave 86 isolates in 147 patients (58.5%) when whole blood was cultured. Of these 147, 99 had clot cultures performed, with 93 positive results (93%), after removal of the serum and dissolution of the clot with streptokinase (6). None had positive blood cultures and negative clot cultures. The 54 patients with negative cultures, either blood or clot, were diagnosed by unequivocal clinical evidence of enteric fever and confirmed by serological evidence of rising antibody titers. Many years' experience of this last technique using 15 ml of bile salt broth containing 100 U of streptokinase per ml have confirmed the value of the method. In general, physical methods of clot lysis, e.g., glass beads and metal filtration sieves, have proved unsatisfactory or dangerous in our experience.

Even in severe bacteremic states, the number of organisms per milliliter of blood is small, often less than 10 organisms per ml (3, 7; K. C. Watson, M.D. thesis, University of Aberdeen, Scotland). Since *S. typhi* is nonexacting in its cultural re-

quirements, poor isolation rates stem either from inadequate volumes of blood being sampled or from death of organisms in culture bottles before growth occurs, due to inadequate dilution of bactericidal serum factors.

An experimental study was carried out to determine the effect of media and blood volumes, to compare results of conventional blood culture systems and those from experimentally infected blood clots, and to consider the effect of serum on organism isolation. The application of whole-blood and blood clot culture systems to isolation of *S. typhi* from clinical cases is also discussed.

MATERIALS AND METHODS

***S. typhi* strains.** Experiments were done either with strains of *S. typhi* freshly isolated from cases of typhoid fever in King Edward VIII Hospital, Durban, Natal, or with the stock laboratory strain *S. typhi* Ty2 (NCTC 8385). Strains were maintained on nutrient agar slopes at room temperature.

Culture medium. Standard 0.5% bile salt broth containing 5 g of sodium taurocholate in 1 liter of sterile nutrient broth (pH 7.6) and autoclaved at 108°C for 15 min was employed. Blood cultures were done by adding 8.0 ml of blood from patients to varying volumes of medium in 120-ml sterile glass bottles. Containers were incubated at 37°C and subcultured daily for 1 week onto Wilson and Blair bismuth sulfite medium (9). The medium was freshly prepared every 3 days from solutions kept at 4.0°C, and plates

were stored at this temperature. The solutions are stable for several months. Negative cultures were then subcultured weekly for 6 weeks before discarding.

Isolation of *S. typhi* was carried out from blood clot as described before (6). Briefly, the clots from 5.0 to 8.0 ml of blood were added after retraction to 15 ml of bile salt broth containing 100 U of streptokinase (Varidase, Lederle, American Cyanamid Co., N.Y.) dissolved in 0.5 ml of sterile 0.85% saline. This produces rapid lysis and release of trapped organisms. Streptokinase has no harmful effects on *S. typhi*. Subcultures were plated as above.

Preparation of artificially infected clots. Overnight nutrient agar slope cultures of *S. typhi* were prepared by inoculating each slope with four 0.02-ml volumes from a 50-drop/ml pipette of an 8-h horse digest broth culture (1). Growth was washed off the slopes with sterile 0.85% saline containing 5% nutrient broth. Suitable 10-fold dilutions were then made in the same medium, and plate counts were performed (2).

Dilutions containing approximately 25 to 30 organisms per ml were pipetted in 1.0-ml volumes into sterile tubes (78 by 13 mm). Four milliliters of blood freshly drawn from a volunteer was then added, and the contents were mixed with gentle rotation until clotting occurred. The tubes were stood at 4.0°C for 2 h, and the separated serum was removed. The clots were decanted into sterile petri dishes and bisected by means of two sterile microscope slides. A fresh plate and slides were used for each clot. Each half clot was then incubated as described below. Clotted specimens from patients with clinical enteric fever were dealt with similarly. A series of preliminary quantitative experiments was carried out to show that the method of artificial clot preparation resulted in even distribution of organisms throughout the clots. This was done by dividing clots into four equal parts, lysing with streptokinase, and performing plate counts.

Immune and nonimmune sera. Immune sera were obtained from patients in the convalescent phase of disease and had O and H agglutinin titers greater than 1,280. Nonimmune sera were obtained from normal healthy volunteers with no history of past enteric infection or of immunization with vaccine containing *S. typhi*, *S. paratyphi* A, or *S. paratyphi* B. O and H titers were less than 20. Heat inactivation of serum was carried out at 56°C for 20 min.

RESULTS

Dilution effect in blood culture. Varying volumes of fresh blood obtained from volunteers who had previously been immunized with TAB (typhoid-paratyphoid A-paratyphoid B) vaccine and whose sera contained O and H antibodies to *S. typhi* were added to 5.0-ml amounts of bile salt broth containing approximately 25 to 30 *S. typhi* organisms in sterile 28-ml glass screw-capped bottles and incubated at 37°C. Daily subcultures were made onto bismuth sulfite medium. Results are detailed in Table 1 for one representative experiment.

Table 1 clearly indicates the inhibiting effect of final serum concentrations greater than about

1/8. This confirms the need to ensure that adequate volumes of medium are used in blood culture systems if negative results are to be avoided. Similar results were obtained when the volume of blood was kept constant and the medium volume varied. When 8.0 ml of whole blood was added to 100, 75, or 50 ml of medium containing approximately 20 viable organisms, growth was obtained on subculture after 24 h. However, with medium volumes of 15, 25, and 35 ml, no growth was evident after 14 days of incubation. Although these findings suggested that 50 ml of medium was adequate for 8 ml of blood, our comparative findings for whole-blood culture and clot culture in clinical cases of enteric fever did not bear this out, presumably because of very low degrees of bacteremia in some patients (see below).

Effect of clot lysis on isolation of *S. typhi*.

Table 2 illustrates the results obtained with 50 bisected clots, where one half of each clot was incubated in streptokinase bile salt broth and the other half in bile salt broth alone.

Two points are apparent from Table 2. Of the 50 half clots, 16 (32%) incubated with bile salt broth alone failed to grow after 14 days of incubation, and 12 of the 34 positive cultures (35%) required 3 or more days of incubation before becoming positive. Where the corresponding halves of the same clots were lysed by streptokinase, all 50 were positive within 48 h of incubation. It is unlikely that the negative results were due to restricted distribution of organisms within the clots.

Effect of immune and nonimmune sera on isolation of *S. typhi* from artificially infected clots. Artificially infected clots were prepared by adding 4.5 ml of blood from an immune or nonimmune donor to 0.5 ml of a suspension of *S. typhi* containing approximately 30 organisms. After bisection of the clots, one half of each was added to 10 ml of streptokinase bile salt broth along with either the total volume of serum originally present or an equivalent volume of sterile 30% bovine serum albumin. Results after incubation for 14 days are shown in Table 3.

In the experiment where the serum was discarded, all 28 clots made from a nonimmune donor grew *S. typhi* within 24 h (Table 3). Where the serum came from an immune donor, two clots were negative after 14 days. We have shown previously that organisms trapped in clots may be killed off by the residual small amounts of serum that are still present and which are not diluted out till lysis occurs (K. C. Watson, M.D. thesis). Those half clots incubated with non-immune serum yielded 17 positive cultures out of the 28 (60.7%), as compared with only 7 out of

TABLE 1. *Effect of varying blood volumes with constant medium volume on recovery of S. typhi*^a

Medium volume (ml)	Blood volume (ml)	Approx plasma dilution	Days of incubation							
			1	2	3	4	5	6	7	14
5.0	0.5	1:22	+	+	+	+	+	+	+	+
5.0	1.0	1:12	+	+	+	+	+	+	+	+
5.0	1.5	1:8.5	—	—	—	+	+	+	+	+
5.0	2.0	1:7	—	—	—	—	—	—	—	—
5.0	2.5	1:6	—	—	—	—	—	—	—	—
5.0	3.0	1:5.5	—	—	—	—	—	—	—	—
5.0	4.0	1:4.5	—	—	—	—	—	—	—	—
5.0	5.0	1:4	—	—	—	—	—	—	—	—

^a *S. typhi* Ty2. Number of organisms per dilution, approximately 25. +, Positive isolation; —, negative isolation.

TABLE 2. *Effect of streptokinase on recovery of S. typhi from artificially infected and bisected blood clots*^a

No. of days of incubation	Half clots in streptokinase bile broth		Half clots in bile broth alone	
	Positive	Negative	Positive	Negative
1	43	7	22	28
2	50	0	22	28
3	—	—	24	26
4	—	—	30	20
8	—	—	32	18
14	—	—	34	16

^a *S. typhi* strain locally isolated (no. 1321). Number of clots prepared, 50. —, Not cultured on this day.

51 (13.7%) where clots were prepared from an immune donor and incubated with serum; these results exemplify the bactericidal effect of antibody and complement on gram-negative organisms.

Effect of fresh and heated immune serum on *S. typhi*. Five-milliliter infected clots were prepared as above, using an immune donor. Serum was removed after 2 h at 4.0°C from half the infected clots and heat inactivated as described. Each clot was then bisected. Half of each was incubated in 15 ml of streptokinase bile salt broth. The other half was incubated along with all the serum in 10 ml of streptokinase bile salt broth, and subcultures were made as before. Results showed that of the 16 half clots incubated with inactivated serum, 14 were positive after 24 h of incubation, and 2 were still negative after 14 days. However, of the 16 incubated with fresh serum only one gave a positive result after 14 days of incubation. All 32 half clots incubated without serum gave positive cultures after 1 day of incubation.

Isolation of salmonellae from patients with clinical enteric fever. Concurrently with these laboratory studies, the isolation of salmonellae from patients with clinical enteric fever was studied. In a series of 152 patients with

TABLE 3. *Effect of immune and nonimmune serum on isolation of S. typhi from artificially infected blood clots*^a

Serum	No. of days of incubation	Culture results with:			
		Serum present		Serum removed	
		Positive	Negative	Positive	Negative
Immune	1	3	48	48	3
	2	3	48	49	2
	7	7	44	49	2
	14	7	44	49	2
Nonimmune	1	17	11	28	0
	2	17	11	—	—
	7	17	11	—	—
	14	17	11	—	—

^a Approximately 30 organisms per clot. Clots incubated in streptokinase bile salt broth. *S. typhi* strain locally isolated (no. 386). —, Not subcultured on this day.

unequivocal clinical enteric fever seen during the first 10 days of illness, the residual clots obtained from 8.0-ml volumes of blood were cultured after discarding the serum. Of 48 incubated in bile salt broth, 36 were positive (75%) after 21 days of incubation. Of these, 25% were positive after 1 day of incubation, and 45% were positive after 2 days. Of the remaining 104 incubated in streptokinase bile salt broth, 98 (94%) were positive at 21 days. Of these, 52% were positive after 1 day and 85% were positive after 2 days of incubation. These findings are detailed more clearly in a series of 18 patients, where the blood clots were bisected, half were incubated in bile salt broth alone, and the other half were incubated in streptokinase bile salt broth (Table 4).

All of the 18 half clots in streptokinase medium were positive and all except one were positive within 72 h. However, one-third of the corresponding half clots incubated in bile salt broth alone failed to grow, and three of them required 5 to 8 days to become positive.

Medium volume in blood cultures from

patients. Two volumes of bile salt broth were compared, 35 ml and 50 ml, following the experimental findings that 50 ml of medium might be adequate for whole-blood cultures. Blood volumes of 8.0 ml from clinical patients were cultured. The first series contained 104 patients and the second contained 210. At the same time, replicate 8.0-ml volumes were collected for clot culture in streptokinase broth. Results are detailed in Table 5.

The percent isolation rate for whole-blood culture increased from 50 to 64% when the medium volume was increased from 35 to 50 ml, and the mean time of incubation required for a positive result dropped from 4.8 to 2.0 days. This compares with a 93% isolation rate and a mean incubation time of 1.9 days for all 314 patients

when a similar volume of clotted blood was cultured.

DISCUSSION

Apart from a suitable culture medium, there are two essential requirements for isolation of salmonellae from blood. These include an optimum sample volume to take account of the low level of bacteremia and a method that will prevent killing of organisms by serum factors, especially antibody and complement. In general, a sample volume of 8 to 10 ml is almost always adequate. Few negative results are to be expected from random distribution of organisms when this is done.

If whole blood is to be cultured, it is essential to prevent bactericidal serum effects either by adequate dilution of the sample in an adequate medium volume or by inhibition of serum bactericidal factors. Poor cultural results quoted in the literature appear to be almost entirely due to a failure to recognize that rapid killing of small numbers of bacteria will occur unless this is done. This applies particularly where patients are first seen during the second and subsequent weeks of illness, when antibody is being freshly produced. The addition of complement-destroying agents such as sodium polyanetholsulfonate (Liquoid) may be helpful, but not all organism killing is due to antibody- and complement-mediated mechanisms.

The comparative results in Table 5 indicate that 50 ml of medium with 8 ml of blood is inadequate for whole-blood cultures. Our own further investigations have shown that somewhere in excess of 100 ml and preferably nearer 200 ml of medium is needed for 8 ml of blood if comparable isolation rates are to be obtained for blood clot and whole-blood cultures. In a series of studies of 100, 150, and 200 ml of medium for 8 ml of blood for whole-blood cultures, the isolation rates compared to those from blood clot were 86, 94, and 100%, respectively. However,

TABLE 4. Culture results from bisected blood clots in enteric fever^a

Patient no.	Days of incubation for positive isolation in:		Organism
	Streptokinase broth	Bile broth	
1	1	1	<i>S. typhi</i>
2	1	1	<i>S. typhi</i>
3	1	3	<i>S. paratyphi A</i>
4	1	4	<i>S. typhi</i>
5	1	neg. at 14	<i>S. typhi</i>
6	2	2	<i>S. typhi</i>
7	2	2	<i>S. typhi</i>
8	2	2	<i>S. typhi</i>
9	2	2	<i>S. typhi</i>
10	2	neg. at 14	<i>S. typhi</i>
11	2	neg. at 14	<i>S. typhi</i>
12	3	3	<i>S. typhi</i>
13	3	5	<i>S. paratyphi A</i>
14	3	5	<i>S. typhi</i>
15	3	neg. at 14	<i>S. typhi</i>
16	3	neg. at 14	<i>S. typhi</i>
17	3	neg. at 14	<i>S. typhi</i>
18	4	8	<i>S. typhi</i>
Percent positive	100	67	

^a Half of each clot was incubated in bile broth and half was incubated in streptokinase bile broth.

TABLE 5. Comparison of 35- and 50-ml volumes of culture medium for whole-blood cultures (8 ml of blood) and isolation from blood clot in clinical cases of enteric fever

No. of patients	Culture and medium ^a	Vol of medium (ml)	Positive isolation	Negative isolation	% positive	Avg time of incubation for positive result (days)
104	8 ml of whole blood in BSB	35	52	52	50	4.8
	Blood clot from 8 ml of blood in SKBSB	15	98	6	94	1.9
210	8 ml of whole blood in BSB	50	135	75	64	2.0
	Blood clot from 8 ml of blood in SKBSB	15	193	17	92	1.9

^a BSB, Bile salt broth; SKBSB, streptokinase bile salt broth.

such large volumes may present problems, especially in developing areas where the disease is common, in terms of cost of medium and necessary incubator facilities to deal with large numbers of cultures. Isolation from blood clot is far more reliable, provided rapid lysis occurs, since organisms trapped within the clot are susceptible to serum factors adsorbed to the fibrin meshwork (K. C. Watson, M.D. thesis). We have utilized the method of lysis with streptokinase in bile salt broth in a series of almost 5,000 cases of enteric fever over a period of 20 years and have been impressed by the high isolation rate. As shown in Table 5, this is of the order of 92 to 94%. The facility of using only 15 ml of medium for blood clot culture from 8 ml of blood is an enormous saving. We have also found that it is easy to spot-inoculate 25 subcultures onto one petri plate of Wilson and Blair bismuth sulfite medium by using an illuminated ruled template. We recommend that the clot technique has many advantages over conventional whole-blood culture, both in reliability and in cost.

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