Rapid, Sensitive Recovery of Recombinant Attenuated \textit{Salmonella enterica} Serovar Typhi Vaccine Strains from Human Blood

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Prior to initiating a phase 1 dose escalation trial of the safety and immunogenicity of live, oral, recombinant, attenuated \textit{Salmonella enterica} serovar Typhi (S. Typhi) vector strains in human subjects, the suitability of conventional blood culture procedures to rapidly and reliably detect the organisms in human blood was investigated. Blood culture specimens, with and without added growth supplements, were inoculated with study organism concentrations ranging from approximately 300 to as few as 1 to 2 CFU/10 ml culture and processed in a Bactec 9240 fluorescent series aerobic blood culture system. All cultures seeded with >6 CFU and 93% of cultures seeded with ~1 to 2 CFU were identified as positive for microbial growth within 44 h of incubation. The results were within the performance standard of ≤5 days to detection that is expected for Gram-negative cultures seeded at 10 to 50 CFU/vial. Recovery of test organisms from blood culture was not improved by the addition of supplements, but cultures with added supplements were identified positive an average of 5 h sooner than those without added supplements. Reliable detection of the investigational vaccine strains at <1 CFU/ml of blood within 2 days in conventional blood culture without added supplements allowed for shortened confinement time of study volunteers without compromising subject safety.

The ability to sensitively and rapidly monitor for bacteremia caused by live oral vaccine organisms is crucial for the evaluation of the safety of investigational vaccines in human subjects during clinical trials. Prior to initiating a phase 1 dose escalation trial of the safety and immunogenicity of live, oral, recombinant, attenuated \textit{Salmonella enterica} serovar Typhi (S. Typhi) vector strains in human subjects, the suitability of conventional blood culture procedures to identify bacteremia caused by these investigational organisms was investigated.

Blood culture is generally considered the standard method for diagnosis of typhoid bacteremia but has been shown to detect only 40 to 70% of typhoid patients (1). In a quantitative study of bacteremia in typhoid fever patients, 50% of patients identified by positive blood culture had less than 1 CFU/ml of blood (2), illustrating the need for highly sensitive methods of detection. Rubin et al. (1) described methods to improve detection of S. Typhi in blood by use of a radiolabeled DNA probe specific for the genes encoding the Vi antigen present in concentrated blood samples. These methods were shown to detect <1 CFU/ml of S. Typhi in bacteremic typhoid patients; however, these methods are not currently applied in most clinical laboratories.

Advances in blood culture technology in the clinical laboratory have greatly enhanced the speed and sensitivity of detection of a broad range of clinically significant pathogens in blood (3). The current standard, Bactec Standard/10 Aerobic/F culture vials, together with Bactec 9240 brand fluorescent series instruments (Becton, Dickinson Diagnostic Instrument Systems, Sparks, MD), provide a sensitive, continuous monitoring blood culture system for aerobic culture and recovery of bacteria, yeast, and fungi from blood (4) and are routinely used in 60 to 70% of clinical labs (Becton, Dickinson Diagnostic Instruments Systems, Sparks, MD, personal communication). For ATCC reference and wild-type Gram-negative organisms seeded at 10 to 50 CFU/vial, this blood culture system has an expected time to detection of ≤5 days (5). Comparative clinical studies have shown that an incubation period of more than 5 days does not significantly improve recovery (4, 6). Therefore, it is standard clinical practice that blood cultures be monitored for 5 full days before a report of negative is generated.

Our phase 1 study protocol specified that volunteers be confined to an inpatient clinical facility for observation and monitoring after receipt of the investigational vaccines. Blood cultures would be collected twice daily for 7 days after vaccination to monitor for potential typhoid bacteremia. Two negative blood cultures on the 7th day postvaccination were required for release of subjects from confinement. Since clinical blood cultures are monitored for 5 full days before they are reported as negative, study volunteers could be confined in a clinical research facility for up to 2 weeks after vaccination pending issue of final blood culture results.

The ability of routine clinical blood culture to detect the investigational S. Typhi organisms was unknown. The vaccine strains used in this study are attenuated, have nutritional limitations, and have been shown to readily succumb to the bactericidal components in fresh human blood (7). Preliminary observations of enhanced survival after 1 h of incubation in blood culture with added supplements, targeted to support growth and fitness of the attenuated strains, compared to nonsupplemented blood culture (data not shown), suggested a possible benefit of additional suppl-
mments to blood culture vials for survival and growth of these inves-
tigational strains.

The objectives of this laboratory study were 2-fold. The first objective was to evaluate the survival, time to positive culture (TTP), and limits of detection in human blood of three investigational, recombinant, attenuated S. Typhi vector strains expressing a gene for *Streptococcus pneumoniae* surface protein antigen PspA (RASV-Sp) (7, 8, 9). Testing was done using the standard Bactec 9240 fluorescent series aerobic blood culture system. The second objective was to determine if recovery or time to detection could be improved by the addition of supplements to support rapid growth of the test organisms in cultures seeded with the lowest possible inoculum (~1 to 2 CFU/vial). These determinations would provide guidance as to the ability of standard clinical blood culture procedures to reliably and rapidly identify bacteremia caused by the attenuated investigational organisms and if supplements were needed to achieve or enhance this capability.

**MATERIALS AND METHODS**

**Test organisms.** Three RASV-Sp strains containing plasmid pYA4088, specifying synthesis of the PspA antigen, were evaluated. These strains differ in parent strain source and RpoS status and have various requirements for optimal growth in culture (7, 8, 9).

\(^{\text{9633}}(\text{pYA4088})\) (S. Typhi ISP1820) is a live, recombinant, attenuated strain with genotype \(\Delta_{\text{Pcrp527}}^{\text{tviABCDE10}}\) \(\text{araC}^{\text{PBAD}}\) \(\text{crp}^{\text{crp}}\); \(\text{TT} \) \(\text{arac}_{\text{PBAD}}\) for \(\Delta_{\text{pmm-2426}}^{\text{gmd-fcl-26}}\) \(\Delta_{\text{opfB1925}}^{\text{trela1988}}\) araC \(\text{PBAD}\) lacI TT \(\Delta_{\text{tami2E25}}^{\text{lami2BAD32}}\) \(\Delta_{\text{vbabcde10}}^{\text{vbabcde10}}\) \(\Delta_{\text{dada33}}^{\text{dada33}}\).

\(^{\text{9639}}(\text{pYA4088})\) (S. Typhi Ty2 RpoS\(^{+}\)) is a live, recombinant, attenuated strain with the genotype \(\Delta_{\text{Pcrp527}}^{\text{tviABCDE10}}\) \(\text{araC}^{\text{PBAD}}\) \(\text{crp}^{\text{crp}}\); \(\text{TT} \) \(\text{arac}_{\text{PBAD}}\) for \(\Delta_{\text{pmm-2426}}^{\text{gmd-fcl-26}}\) \(\Delta_{\text{opfB1925}}^{\text{trela1988}}\) araC \(\text{PBAD}\) lacI TT \(\Delta_{\text{tami2E25}}^{\text{lami2BAD32}}\) \(\Delta_{\text{vbabcde10}}^{\text{vbabcde10}}\) \(\Delta_{\text{dada33}}^{\text{dada33}}\).

\(^{\text{9640}}(\text{pYA4088})\) (S. Typhi Ty2 RpoS\(^{-}\)) is a live, recombinant, attenuated strain with the genotype \(\Delta_{\text{Pcrp527}}^{\text{tviABCDE10}}\) \(\text{araC}^{\text{PBAD}}\) \(\text{crp}^{\text{crp}}\); \(\text{TT} \) \(\text{arac}_{\text{PBAD}}\) for \(\Delta_{\text{pmm-2426}}^{\text{gmd-fcl-26}}\) \(\Delta_{\text{opfB1925}}^{\text{trela1988}}\) araC \(\text{PBAD}\) lacI TT \(\Delta_{\text{tami2E25}}^{\text{lami2BAD32}}\) \(\Delta_{\text{vbabcde10}}^{\text{vbabcde10}}\) \(\Delta_{\text{dada33}}^{\text{dada33}}\).

**Preparation of test organisms for blood culture.** Overnight cultures of \(^{\text{9633}}(\text{pYA4088})\), \(^{\text{9639}}(\text{pYA4088})\), and \(^{\text{9640}}(\text{pYA4088})\) were prepared from frozen vaccine seed stocks and incubated at 37°C with aeration in a phosphate-buffered, vegetable-based proprietary medium (KT broth). The growth medium for all RASV-Sp strains was supplemented with 0.1% (−) mannose, 0.05% l- (+)-arabinose, 20 μg/ml l-tryptophan, and 22 μg/ml l-cysteine HCl. Strains \(^{\text{9633}}(\text{pYA4088})\) and \(^{\text{9640}}(\text{pYA4088})\) also received 0.1% d- (+)-arabinose in all growth media (supplements were obtained from Sigma, St. Louis, MO, USA). The following day, overnight cultures were subcultured to fresh medium, supplemented as described above, and incubated for approximately 4 h at 37°C with aeration of 200 rpm until late-log-phase growth (an optical density at 600 nm of ~2.0, which corresponds to a culture density of 1 × 10^8 CFU/ml). Serial dilutions of each culture were prepared in phosphate-buffered saline (PBS) to generate inocula for blood culture vials. Ten-fold dilutions were prepared by adding 0.5 ml of harvested culture to 4.5 ml of PBS in sterile-capped 16- by 150-mm glass tubes, mixing, and transferring in series to achieve an estimated culture density of 1,000 and 100 CFU/ml in the 6th and 7th tubes, respectively. Further downstream dilutions were made in larger volumes to prepare CFU/ml inocula ranging from approximately 200 to ~1 to 2 CFU/ml for seeding replicates of 10-ml test blood culture specimens, representing a bacterial concentration range of approximately 20 to 0.1 CFU/ml of blood collected in a clinical test sample.

**Preparation of Bactec 9240 culture vials.** Bactec Standard/10 Aerobic/F culture vials (here referred to as Bactec vials or vials) containing 40 ml F medium comprised of enriched soybean-casein digest broth dispensed with added CO\(_2\) were used for all experiments. Five normal, healthy volunteers (3 male and 2 female; age, 20 to 58) gave informed consent and provided blood for the experiments, in compliance with pro-
the first experiments, 24 blood culture vials (eight for each of 3 RASV-Sp strains) were prepared with fresh blood from a single volunteer. Suspensions of each RASV-Sp subculture were prepared as described above to approximate 200 CFU/ml, and serial 1:3 dilutions were prepared in PBS to generate suspensions of approximately 66 CFU/ml, 22 CFU/ml, and 7 CFU/ml.

Because the RASV-Sp strains are tryptophan and cysteine auxotrophs and possess a ΔP araC araC P araC araC P mutation which imposes a growth defect in the absence of arabinose, we tested to see if the addition of these supplements to the culture medium would improve recovery of the RASV-Sp strains. Mannose was supplied to allow for complete lipopolysaccharide (LPS) O-antigen synthesis during blood culture.

For each RASV-Sp strain, 4 vials were prepared with added supplements and 4 without added supplements. One milliliter of each seed suspension was inoculated into 1 supplemented and nonsupplemented vial before transfer to Quest Diagnostics for processing.

After confirmation that test cultures could be detected at higher concentrations, experiments were performed to test the RASV-Sp strains in blood culture at the lowest possible inoculum of 1 CFU/vial. Twenty blood culture vials for each of three RASV-Sp strains were prepared (one strain per day on three consecutive days), using fresh blood from a different individual each day. For each RASV-Sp series, 10 vials were prepared with added supplements and 10 without added supplements. Suspensions of RASV-Sp strains were prepared as described above to approximately 10 CFU/ml and 1 CFU/ml. Three of each supplemented and nonsupplemented vials for each strain were inoculated with a 1-ml suspension containing ~10 CFU as low positive controls. Seven of each supplemented and nonsupplemented vials for each strain were inoculated with 1 ml suspension containing ~1 CFU.

Culture vials were transferred to Quest Diagnostics for processing as described above. As an internal lab control for the presence of bacteria in ~1 CFU/ml seed suspensions, 1 ml of each RASV-Sp strain suspension containing ~1 CFU/ml was added to 3 tubes each of 40 ml phosphate-buffered KT broth at the same time the Bactec vials were inoculated. These permissive control cultures were retained and incubated at 37°C in the SLU research lab and observed for visible growth after incubation for 36 to 72 h.

**Quality assurance results.** Quality assurance negative-control plates for all experiments showed no evidence of contaminating organisms on plates spread with primary medium, subculture medium, or PBS used in any RASV-Sp strain culture and inoculum preparations. All seed preparations of RASV-Sp strains spread to TSA, MMA, and MAMA showed the typical strain phenotype, were Gram negative, and were positive for agglutination with *Salmonella* O, factor 9, group D antiserum, confirming RASV-Sp strain identities. All blood cultures giving a positive signal and subcultured to blood agar at Quest Laboratory were recovered and confirmed as pure Gram-negative cultures with the typical phenotype for the RASV-Sp strains. A subset of 9 cultures (three of each RASV-Sp strain) was further tested for RASV-Sp strain identity and confirmed positive for agglutination with *Salmonella* O, factor 9, group D antiserum. Internal lab controls of ~1 CFU/ml RASV-Sp seed suspensions when inoculated into permissive KT broth media showed growth by visual cloudiness in 8 of 9 cultures within 72 h of incubation at 37°C.

**Calculated CFU inoculated to blood culture vials.** Counts of titrated and plated RASV-Sp seed preparations showed that high-dose test vials were inoculated with average titrated doses of 272, 91, 30, and 10 CFU/vial, representing concentrations in blood of 27, 9, 3, and 1 CFU/ml. Low-dose vials were seeded with 2.3, 1.6, and 1.2 CFU/vial for χ9633(pYA4088), χ9639(pYA4088), and χ9640(pYA4088), respectively, achieving the minimum possible seed doses of ~1 to 2 CFU/vial, equivalent to ~0.1 to 0.2 CFU/ml of blood for all three strains.

**Recovery and TTP of RASV-Sp in blood culture.** A rapid and high rate of recovery of viable test organisms from blood cultures seeded with any of the RASV-Sp strains was observed, with and without added supplements. Eighty-one of 84 blood culture vials tested in combined experiments seeded with any RASV-Sp strain in doses ranging from 1.2 to 355 CFU/10 ml of blood were detected as positive for live organisms within 44 h of culture, giving an overall 96% recovery rate in less than 2 days of incubation. All 42 vials seeded with ≥6 CFU/vial were detected within 39 h of incubation. At the lowest inoculum of ~1 to 2 CFU/vial, 39 of 42 cultures (93%) were recovered and were all detected within 44 h of incubation. Since the seed suspensions used to inoculate the lowest inoculum blood cultures contained only ~1 to 2 CFU/ml, it is possible that no organisms were present in the 1 ml inoculum used to seed the 3 vials that remained negative after 5 days incubation, and these results may have been true negatives. Using Poisson distribution analysis, one would expect two to three negative cultures.

**TABLE 1 Recovery and time to positive culture of the RASV-Sp strain in blood cultures without added supplements for vials seeded with ≥6 CFU**

| Strain   | Calculated no. of CFU/vial | TTP (h) | Avg TTP (h) (95% CI) |
|----------|----------------------------|---------|----------------------|
| χ9633(pYA4088) | 355 | 32.0 | 33.3 (31.0–35.5) |
| 118      | 30.2 |         |                      |
| 39       | 32.7 |         |                      |
| 13       | 37.7 |         |                      |
| 12       | 35.0 |         |                      |
| 12       | 32.5 |         |                      |
| 12       | 32.9 |         |                      |
| χ9639(pYA4088) | 164 | 23.5 | 28.9 (24.4–33.3) |
| 54       | 26.0 |         |                      |
| 18       | 27.5 |         |                      |
| 6        | 38.5 |         |                      |
| 8        | 27.1 |         |                      |
| 8        | 30.8 |         |                      |
| 8        | 28.6 |         |                      |
| χ9640(pYA4088) | 299 | 26.3 | 28.6 (24.2–32.9) |
| 100      | 28.8 |         |                      |
| 33       | 30.4 |         |                      |
| 11       | 33.9 |         |                      |
| 6        | 19.3 |         |                      |
| 6        | 29.9 |         |                      |
| 6        | 31.4 |         |                      |

*Calculated numbers of CFU/vial are based on plated titrations of preparations used to seed blood culture vials.

+ Time to positive culture (TTP) in hours from insertion in the Bactec blood culture instrument until generation of a positive signal.

*Average time to positive culture in hours, with 95% confidence intervals (CI).
TABLE 2 Recovery and time to positive culture of RASV-Sp in blood cultures without added supplements for vials seeded with \(-1\) to \(2\) CFU

| Strain            | Calculated no. of CFU/vial \(^a\) | TTP (h) \(^b\) | Avg TTP (h) \(^c\) (95% CI) |
|-------------------|------------------------------------|--------------|--------------------------|
| \(\chi9633(pYA4088)\) | 2.3                                 | 43.6         | 39.6 (35.2–44.1)         |
|                   | 2.3                                 | Neg          |                          |
|                   | 2.3                                 | 32.2         |                          |
|                   | 2.3                                 | 41.1         |                          |
|                   | 2.3                                 | 41.6         |                          |
|                   | 2.3                                 | 42.1         |                          |
|                   | 2.3                                 | 37.1         |                          |
| \(\chi9639(pYA4088)\) | 1.6                                 | 31.6         | 30.5 (28.8–32.2)         |
|                   | 1.6                                 | 27.3         |                          |
|                   | 1.6                                 | Neg          |                          |
|                   | 1.6                                 | 31.1         |                          |
|                   | 1.6                                 | 30.5         |                          |
|                   | 1.6                                 | 31.1         |                          |
|                   | 1.6                                 | 31.3         |                          |
| \(\chi9640(pYA4088)\) | 1.2                                 | 34.9         | 33.6 (31.3–35.8)         |
|                   | 1.2                                 | 34.2         |                          |
|                   | 1.2                                 | 33.2         |                          |
|                   | 1.2                                 | 30.9         |                          |
|                   | 1.2                                 | 31.4         |                          |
|                   | 1.2                                 | 32.4         |                          |
|                   | 1.2                                 | 37.9         |                          |

\(^a\) Calculated numbers of CFU/vial are based on plated titrations of preparations used to seed replicate blood culture vials of each strain. Inocula for each strain were prepared separately, on 3 consecutive days, using blood from a different individual each day.

\(^b\) Time to positive culture (TTP) in hours from insertion in the Bactec blood culture instrument until generation of a positive signal. Neg, negative culture after 5 full days of incubation.

\(^c\) Average time to positive culture in hours, with 95% confidence intervals (CI).

Results comparing TTP for all 81 recovered cultures with and without added supplements, and seeded with a range from 1.2 to 355 CFU/vial, are shown together in Fig. 2. The average time to detection for 41 cultures with added supplements was 27 h (range, 19.6 to 35.8), compared to an average of 32 h (range, 19.3 to 43.6) to detection for the 40 cultures without added supplements. Overall, an average reduction in TTP of 5 h was observed in vials with added supplements, which is a 16% reduction in TTP compared to that of vials without added supplements \((P < 0.001)\).

DISCUSSION

The manufacturer’s expected performance parameters using Standard/10 Aerobic/F culture vials are that various ATCC and wild-type organisms, seeded at 10 to 50 CFU/vial, can be detected within 5 days of incubation \((5)\). For the studies reported here, all positive blood cultures, including the cultures seeded with \(-1\) to 2 CFU of RASV-Sp strains/10 ml blood, with or without added supplements, were identified within 2 days of incubation.

Recovery of pathogens in blood culture can be affected by multiple parameters in addition to bacterial concentration, including blood volume, culture medium composition and volume, and incubation time \((10)\). Successful recovery of these attenuated RASV-Sp strains from blood culture without added supplements may be due in part to the enrichment factors present in F medium, which include yeast extract, animal tissue digest, succrose, hemin, menadione, and vitamin B\(_6\) as well as nutritional components available in human blood. RASV-Sp strains are sensitive to inhibiting factors found in fresh blood, including complement components \((7)\). However, the dilution of blood specimens in broth at a ratio of 1:5, as was used in these studies, may mitigate this effect and has been shown to be optimal to support growth of most organisms in blood culture \((10)\). Clinical blood culture media and culture conditions are developed and optimized to recover a broad spectrum of possible disease-causing organisms, including slow-growing, fastidious species. Results reported here for our investigational strains are consistent with other published studies, indicating that incubation time of fewer than 5 days may be sufficient for recovery of many clinically significant organisms \((11, 12, 13, 14)\).

These results demonstrate that conventional blood culture methods, using the Bactec 9240 fluorescent series blood culture system and Standard/10 Aerobic/F culture vials, provided reliable recovery of three recombinant attenuated S. Typhi vector vaccine organisms from human blood inoculated with \(-1\) to 2 CFU/10 ml blood \((<-1\ CFU/ml)\), within 2 days of incubation, and without added supplements. This allows a more accurate evaluation of the clinical behavior of live, attenuated S. Typhi strains and also decreases the risk associated with administering such strains in a clinical trial setting. The addition of supplements provided a small but significant reduction in the time to detection but did not enhance recovery over nonsupplemented cultures. The potential benefit of supplementation in order to achieve more rapid detection of a test organism should be weighed against the risk of introducing contaminants when adding materials to sterile media in culture vials that have been manufactured under highly controlled conditions. Further studies should be undertaken to confirm and extend these findings in other trials and to validate this procedure for clinical use.

The results of these studies provided guidance for conduct of a phase 1 vaccine trial of live oral S. Typhi vaccine strains. Study
volunteers were subjected to fewer days of confinement which, in turn, reduced study costs associated with extended confinements. The sensitivity and rapidity of detection enabled by the Bactec 9240 blood culture system provides an important and broadly available tool to support future studies of the safety of recombinant, attenuated S. Typhi vaccines in human volunteers.

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