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Blood Volume Required for Detection of Low Levels and Ultralow Levels of Organisms Responsible for Neonatal Bacteremia by Use of Bactec Peds Plus/F, Plus Aerobic/F Medium, and the BD Bactec FX System: an *In Vitro* Study

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We used an *in vitro* technique to investigate blood volumes required to detect bacteremia and fungemia with low concentrations of an organism. At 1 to 10 CFU/ml, *Escherichia coli*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Candida albicans*, and *Candida parapsilosis* isolates were detected in volumes as low as 0.5 ml. Detection of *Streptococcus agalactiae* and detection of bacteremia at <1 CFU/ml were unreliable.

Neonatal sepsis continues to be a serious condition. While incidence of early-onset neonatal *Streptococcus agalactiae* infections has decreased, late-onset disease and *Enterobacteriaceae* sepsis rates remain stable (1–3). Additionally, the frequent use of central venous catheters in premature and ill newborns places them at risk of developing central line-associated bloodstream infections. Coagulase-negative *Staphylococcus* species are a leading causative agent. *Staphylococcus aureus*, *Enterobacteriaceae*, and *Candida* species are reported less frequently (4).

There is evidence to suggest that the likelihood of a blood culture testing positive in a bacteremic neonate is dependent on the volume of blood drawn (5). Accordingly, the College of American Pathologists requires that laboratories have a system to monitor blood culture volumes submitted. To provide guidance around appropriate blood draw volumes in pediatric patients, there are currently four sets of published recommendations. However, when applied to neonates, the recommended volumes vary considerably (Fig. 1) (6–9).

In this study, we used *in vitro* methods to investigate the blood volumes necessary to detect common agents of neonatal sepsis at low levels (1 to 10 CFU/ml) and ultralow levels (<1 CFU/ml). Also, we compared the sensitivity of pediatric Bactec Peds Plus/F media and adult Bactec Plus Aerobic/F media (BD Diagnostics, Sparks, MD).

We used American Type Culture Collection (ATCC) organisms, as summarized in Table 1. These organisms were chosen to represent common agents of neonatal sepsis (10, 11). *S. agalactiae* serotypes Ia and III were chosen, as they are the most prevalent serotypes involved in invasive neonatal *S. agalactiae* infections in the United States (12). After overnight culture on 5% sheep's blood agar and a subsequent subculture, each organism was serially diluted using a digital pipettor to achieve concentrations of 1 to 10 CFU/ml and <1 CFU/ml. Specifically, for each bacterial isolate, a stock suspension was prepared starting with a 1.0 McFarland standard suspension ($\approx 3 \times 10^8$ CFU/ml). Three successive 100-fold dilutions were prepared using sterile saline (Remel, Lenexa, KS) to produce a stock suspension concentration of $\approx 3 \times 10^2$ CFU/ml. With *Candida* species, we started with a 0.5 McFarland standard ($\approx 3 \times 10^6$ CFU/ml) and performed two successive 100-fold dilutions using sterile saline to produce a stock suspension concentration of $\approx 3 \times 10^2$ CFU/ml (13). To produce an experimental source of blood with 1 to 10 CFU/ml, 1 ml of the stock suspension was added to 49 ml of donated, banked, citrated, adult human whole blood that had been collected within the previous 48 h (≈6 CFU/ml). To produce blood with <1 CFU/ml of organism, a 1:10 dilution was performed on the stock solution, then 1 ml of this solution was added to 49 ml of blood, generating

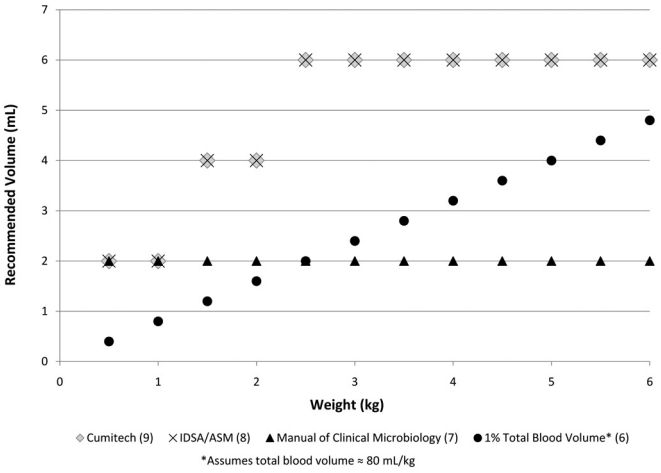

FIG 1 Blood culture volume recommendations by weight in kilograms.

Received 24 June 2015 **Returned for modification** 20 July 2015
Accepted 11 August 2015

Accepted manuscript posted online 19 August 2015

Citation Lancaster DP, Friedman DF, Chiotos K, Sullivan KV. 2015. Blood volume required for detection of low levels and ultralow levels of organisms responsible for neonatal bacteremia by use of Bactec Peds Plus/F, Plus Aerobic/F medium, and the BD Bactec FX system: an *in vitro* study. J Clin Microbiol 53:3609–3613. doi:10.1128/JCM.01706-15.

Editor: K. C. Carroll

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TABLE 1 Organism suspension colony counts

Organism	ATCC strain	Organism suspension, CFU/ml	
		<1	1–10
E. coli	25922	0.4	4.8
S. agalactiae	12386	0.2	3.4
S. agalactiae (serotype 1a)	BAA 1138	0.4	4.6
S. agalactiae (serotype III)	12403	0.2	4.2
S. aureus (MSSA)[a]	25923	0.8	6.2
S. aureus (MRSA)[b]	BAA 1556	0.2	6.4
S. epidermidis	12228	0.6	3.0
L. monocytogenes	BAA 751	0.2	3.0
C. albicans	60193	0.6	3.2
C. parapsilosis	22019	0.8	6.6

[a] MSSA, methicillin-susceptible *S. aureus*.
[b] MRSA, methicillin-resistant *S. aureus*.

a bacterial concentration of ≈0.6 CFU/ml. At each step, suspensions were either vortexed or swirled to ensure homogeneity.

Once the organism suspensions were prepared, colony counts were verified as follows. A 1-ml aliquot of each of the final suspensions of 1 to 10 CFU/ml and <1 CFU/ml was inoculated on to a Mueller-Hinton agar plate with 5% sheep's blood and streaked for quantitation. None of the <1 CFU/ml suspensions yielded growth at this volume. Colony counts are summarized in Table 1. We also inoculated 100 μl of the penultimate suspensions of the <1 CFU/ml preparations (≈30 CFU/ml) to ensure that bacterial cells were present prior to the final 1:50 dilution.

Organism suspensions were then inoculated into Bactec Peds Plus/F and Bactec Plus Aerobic/F bottles at volumes of 0.5, 1, 1.5, 2, and 3 ml in triplicate. A Peds Plus/F and a Plus Aerobic/F bottle each were inoculated with 3 ml of banked blood only (no organism) to serve as negative controls. All bottles were incubated in a Bactec FX instrument for a maximum of 5 days. Positive blood cultures were subcultured to 5% Columbia sheep's blood agar to confirm growth of the appropriate organism. Terminal subcultures were performed on all negative bottles using 200 μl of blood culture broth.

A total of 600 bottles were inoculated with organism suspension. Negative control bottles were negative at 5 days. Terminal subcultures did not yield growth. Organism recovery is summarized in Table 2 . A total of 219 of 300 (73%) Peds Plus/F bottles and 194 of 300 (65%) Plus Aerobic/F bottles were positive ($P = 0.03$). Among the organism/bacterial load/volume triplicate groups in which organism was recovered in 3 of 3 bottles in both bottle types ($n = 234$ bottles qualified), the mean time to detection was 20.4 h for Peds Plus/F and was 21.1 h for Plus Aerobic/F ($P = 0.02$).

At a bacterial load of 1 to 10 CFU/ml, *Escherichia coli*, *Staphylococcus aureus* (methicillin-sensitive *Staphylococcus aureus* [MSSA] and methicillin-resistant *Staphylococcus aureus* [MRSA]), *Listeria monocytogenes*, *Candida albicans*, and *Candida parapsilosis* were recovered in all bottles tested. *S. agalactiae* was detected in 39 of 45 (87%) Peds Plus/F bottles and in 25 of 45 (56%) Plus Aerobic/F bottles. We failed to recover *S. agalactiae* ATCC BAA 1138 consistently, even with 3 ml of blood.

At <1 CFU/ml, the Peds Plus/F recovered *E. coli* in bottles inoculated with ≥1.5 ml of organism suspension; ≥1.0 ml was required for recovery of MSSA, and 3 ml was required for *C. albicans* and *C. parapsilosis*. With Peds Plus/F, the detection of *S. agalactiae*, *L. monocytogenes*, and MRSA was unreliable at all volumes tested at this organism load. The Plus Aerobic/F detected MSSA consistently at volumes of ≥1.5 ml but had poor sensitivity for all other organisms at this organism load.

There is a paucity of data supporting recommended draw volumes in neonates. To ensure that blood culturing does not contribute to the development of iatrogenic anemia, care is required when extrapolating concepts of ideal blood draw volumes from older children to younger ones. In this study, we sought to determine the blood volumes required to reliably detect low levels and ultralow levels of organisms commonly implicated in neonatal bacteremia.

Our data suggest that, if detection of bacteremia at 1 to 10 CFU/ml is the clinical goal, blood draw volumes as low as 0.5 ml may reliably detect most organisms implicated in early-onset neonatal bacteremia. However, detection of certain strains of *S. agalactiae* may require >3 ml. At simulated bacteremia levels of <1 CFU/ml, Peds Plus/F detected the organism with volumes as low as 1 to 1.5 ml for *E. coli*, MSSA, *S. aureus*, and *Staphylococcus epidermidis*. Three milliliters appeared to suffice for the detection of *Candida* species. *S. agalactiae*, MRSA, and *L. monocytogenes* were difficult to detect reliably, even at volumes as high as 3 ml. The Plus Aerobic/F bottles were unreliable in detecting any organism at any volume except for MSSA. Overall, we failed to recover organism in 86 of 120 blood cultures (72%) inoculated with 0.5 ml or 1 ml of blood with an organism load of <1 CFU/ml, suggesting that bacteremia at this level may be missed in significant numbers when small volumes of blood are drawn for culture.

It was previously suggested that neonatal bacteremia is associated with high bacterial loads (14, 15). Kellogg et al. refuted this concept in a study that used a pediatric isolator system and large-volume blood collections (4.5% of total blood volume) to quantify bacteremia in infants at 0 to 2 months of age. They reported that 7 of 15 (47%) cases of *S. agalactiae* and 4 of 11 (36%) cases of *E. coli* bacteremia had bacterial loads of <1 CFU/ml (16). These results are somewhat difficult to interpret, however, as other studies have suggested that bacterial recovery from pediatric isolator tubes may be inferior to broth-based media, such as those used in our study (5, 17). Bacterial inhibition by saponin (the cell lysing agent present in pediatric isolator systems) has been suggested as the culprit (14). Further studies confirming the concept of ultralow level bacteremia in neonates and older children, therefore, would be beneficial. Nevertheless, if detection of bacteremia levels of <1 CFU/ml is desired, blood volumes of >3 ml need to be considered and balanced against the hazard of drawing larger volumes in small neonates.

Brown et al. published a study in 1995 based on the Bactec NR-660 system, which was introduced in 1984 (18). This system used infrared spectrophotometry to detect carbon dioxide produced by growing organisms. *E. coli* and *S. agalactiae* bacteremia was simulated at levels of 0.4, 4, and 40 CFU/ml. Bactec NR-6A bottles were inoculated with volumes of 0.25, 0.5, and 1 ml. At 1 to 10 CFU/ml, *E. coli* was detected in 26 of 26 bottles with 0.5 ml or 1 ml and in 12 of 15 bottles inoculated with 0.25 ml. *S. agalactiae* at 1 to 10 CFU/ml was detected in 10 of 15, 13 of 15, and 15 of 15 bottles inoculated with 0.25, 0.5, and 1 ml of seeded blood. Both *E. coli* and *S. agalactiae* grew inconsistently in bottles inoculated with

TABLE 2 Blood culture bottle yield by organism, volume of blood, level of bacteremia, and bottle type

Organism (ATCC strain)	Volume (ml)	Yield[c] with:			
		Bactec Peds Plus/F		Bactec Plus Aerobic/F	
		<1 CFU/ml	1–10 CFU/ml	<1 CFU/ml	1–10 CFU/ml
E. coli (25922)	0.5	1/3	3/3	1/3	3/3
	1	2/3	3/3	1/3	3/3
	1.5	3/3	3/3	2/3	3/3
	2	3/3	3/3	2/3	3/3
	3	3/3	3/3	2/3	3/3
	Total	12/15	15/15	8/15	15/15
S. agalactiae (12386)	0.5	0/3	2/3	1/3	2/3
	1	0/3	1/3	0/3	2/3
	1.5	2/3	2/3	1/3	3/3
	2	2/3	3/3	1/3	3/3
	3	2/3	3/3	2/3	3/3
	Total	6/15	11/15	5/15	13/15
S. agalactiae (BAA 1138)	0.5	1/3	3/3	0/3	2/3
	1	2/3	3/3	0/3	2/3
	1.5	2/3	3/3	1/3	0/3
	2	2/3	2/3	0/3	2/3
	3	0/3	2/3	0/3	2/3
	Total	7/15	13/15	1/15	8/15
S. agalactiae (12403)	0.5	0/3	3/3	0/3	1/3
	1	0/3	3/3	0/3	0/3
	1.5	1/3	3/3	0/3	1/3
	2	2/3	3/3	2/3	0/3
	3	1/3	3/3	0/3	2/3
	Total	4/15	15/15	2/15	4/15
MSSA[a] (25923)	0.5	1/3	3/3	2/3	3/3
	1	3/3	3/3	2/3	3/3
	1.5	3/3	3/3	3/3	3/3
	2	3/3	3/3	3/3	3/3
	3	3/3	3/3	3/3	3/3
	Total	13/15	15/15	13/15	15/15
MRSA[b] (BAA 1556)	0.5	0/3	3/3	0/3	3/3
	1	1/3	3/3	0/3	3/3
	1.5	1/3	3/3	0/3	3/3
	2	1/3	3/3	1/3	3/3
	3	2/3	3/3	2/3	3/3
	Total	5/15	15/15	3/15	15/15
S. epidermidis (12228)	0.5	1/3	3/3	2/3	3/3
	1	3/3	3/3	2/3	3/3
	1.5	3/3	3/3	2/3	3/3
	2	2/3	2/3	3/3	3/3
	3	3/3	3/3	2/3	3/3
	Total	12/15	14/15	11/15	15/15
L. monocytogenes (BAA 751)	0.5	0/3	3/3	0/3	3/3
	1	0/3	3/3	0/3	3/3
	1.5	0/3	3/3	2/3	3/3
	2	1/3	3/3	2/3	3/3
	3	0/3	3/3	2/3	3/3
	Total	1/15	15/15	6/15	15/15

(Continued on following page)

TABLE 2 (Continued)

Organism (ATCC strain)	Volume (ml)	Yield[c] with: Bactec Peds Plus/F <1 CFU/ml	Bactec Peds Plus/F 1–10 CFU/ml	Bactec Plus Aerobic/F <1 CFU/ml	Bactec Plus Aerobic/F 1–10 CFU/ml
C. albicans (60193)	0.5	1/3	3/3	0/3	3/3
	1	0/3	3/3	0/3	3/3
	1.5	1/3	3/3	0/3	3/3
	2	1/3	3/3	2/3	3/3
	3	3/3	3/3	2/3	3/3
	Total	6/15	15/15	4/15	15/15
C. parapsilosis (22019)	0.5	2/3	3/3	2/3	3/3
	1	1/3	3/3	2/3	3/3
	1.5	2/3	3/3	3/3	3/3
	2	2/3	3/3	2/3	3/3
	3	3/3	3/3	2/3	3/3
	Total	10/15	15/15	11/15	15/15

[a] MSSA, methicillin-susceptible *S. aureus*.
[b] MRSA, methicillin-resistant *S. aureus*.
[c] Number of bottles positive per triplicate.

any volume at levels of <1 CFU/ml. Our findings corroborated these results.

Using *in vitro* methods, we observed superior sensitivity and shorter time to positivity in Peds Plus/F bottles than in adult Plus Aerobic/F media. We are not aware of any other data in the published literature documenting this finding. This may be relevant for pediatric hospital settings that use the Bactec blood culture system.

This study has a number of limitations. First, banked, citrated, whole adult human blood was used as a proxy for fresh blood from a neonate. Citrate may have a subtle inhibitory effect on bacterial growth, which may have led to an overestimation of the volume of blood required to detect bacteremia. Also, plasma proteins, including coagulation factors, complement, and immunoglobulin, may degrade with prolonged storage. Additionally, there have been anecdotal observations of impaired organism recovery in seeded blood cultures when old, expired units of blood are used. We minimized this effect by using blood that was received within 48 h of donation, immediately upon receipt. Second, our findings apply strictly to the Bactec FX system. In a recent study, we demonstrated superior sensitivity and swifter time to detection of bacteremia in Bactec Peds Plus/F media compared to BacT/Alert Pediatric FAN media (19). Required blood volumes, therefore, may vary according to the blood culture system in use. Third, colony counts were performed on the penultimate dilution for blood prepared with bacterial concentrations of <1 CFU/ml. We used this approach, as we could not find an accurate or reliable method to perform colony counts using large volumes of blood, which we anticipated would be required for testing for *S. agalactiae*. Fourth, actual bacterial loads in neonatal bacteremia are unknown. Our decision to examine organism recovery in simulated bloodstream infections with <1 CFU/ml and 1 to 10 CFU/ml was based on the findings of Kellogg et al. (16). Finally, this study used simulation techniques to estimate the volume of blood required to detect bacteremia in neonates. Multicenter, randomized clinical studies involving bacteremic neonates that examine the yield of incrementally increasing blood culture draw volumes in neonates would be ideal and are much needed. While this study does not determine the adequacy or inadequacy of current published pediatric blood draw volume recommendations, our data can potentially be used in conjunction with studies that investigate magnitude of bacteremia in neonates to better understand appropriate blood draw volumes in this population.

In summary, published recommendations for pediatric blood culture volumes vary, and we sought to determine the volumes required to detect organisms implicated in neonatal bacteremia. In an *in vitro* setting, with the Bactec Peds Plus/F system, 0.5 ml appeared sufficient to detect all organisms at a load of 1 to 10 CFU/ml with the exception of *S. agalactiae*. Recovery of *S. agalactiae* varied considerably according to strain. Larger volumes would likely need to be collected if detection of bacteremia at levels of <1 CFU/ml was desired, but this would need to be considered in tandem with the risks associated with increased draw volumes. The Peds Plus/F media recovered organism with superior sensitivity and faster time to detection than the adult Plus Aerobic/F media. Large-scale clinical studies examining recommended volumes using the various blood culture systems would be beneficial.

ACKNOWLEDGMENTS

We thank Deborah Blecker-Shelly for her input into the study protocol. We also thank Pamala Blair and Maria Dirkes from transfusion medicine at CHOP for their assistance in procuring appropriate banked whole blood.

We have no conflicts of interest to declare.

This study was not funded.

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