Effect of delayed vial entry on the recovery and time to positivity of microorganisms from automated blood culture vials in a tertiary care hospital, South India

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Abstract
Recommendations by manufacturers depict that blood culture bottles are to be loaded into the automated machines as soon as possible after the blood collection. But it has been noticed that in peripheral set-ups where round the clock laboratory facility is not available or which are located far from the reference labs, or even in the tertiary care centres, delay occurs prior to the bottle loading into the instruments. In our study, we have compared the effect of pre-incubating the blood culture bottles at various temperatures like 4°C, room temperature and 37°C for various holding times, such as 2, 6, 12 and 24 hours simulating the delay in transport time in BACTEC plus Aerobic and BACTALERT FA plus systems in a tertiary care set up. We included five recent clinical isolates of different microorganisms in our study, such as Staphylococcus aurous, Streptococcus, Escherichia coli, Pseudomonas aeruginosa and Candida albicans. Standard inoculum was prepared for each organism and 3 ml of it was added with 10 ml of citrated human blood and then inoculated into blood culture vial (BACTALERT FA plus and BACTEC Plus). Organism recovery rate, time to detection and rates of false-negativity from both the instruments were evaluated by using seeded blood culture vial and controls with delayed entry. Performance wise analysis showed that the overall isolation rate of the organisms from BACTEC bottles was higher compared to BACTALERT. Also BACTEC showed less average TTD compared to BACTALERT for Pseudomonas aeruginosa, Streptococcus and candida albicans but for E. coli and S. aureus BACTALERT showed earlier detection. To conclude, storage of the inoculated bottles at room temperature gave optimal recovery of the organisms for both BACTEC and BACTALERT systems, if delayed entry is inevitable.

Keyword: Delayed vial entry effect on recovery of microorganisms.

Introduction
Blood culture results are of paramount importance to the clinician in the management of patients with suspected bacteremia. Numerous factors influence the likelihood of detecting bacteremia such as organism load, transport time to laboratory, volume of blood drawn etc.[1–3] Of these factors, the transport time to laboratory is the most important variable responsible for recovery of microorganisms from the blood.[4] After inoculation into blood culture vials which takes place at the patient bedside, vials are then transported to the laboratory and loaded into automated blood culture instruments or conventional incubators. Ideally, the time in transit from the patient to the instrument should be kept at least less than 2 hours. However, delayed vial loading (DVL) i.e. prolonged delays between specimen collection and the loading of the blood culture vials into the instruments has become a common occurrence now a days. This may be due to several factors such as lack of man power for transport and the proliferation of satellite laboratories etc.[4,5]

The delayed vial loading (DVL) may be associated with poor outcome such as false negativity (poor organism recovery) and prolonged time to positivity. The volume of blood also influences the time to positivity (TTP) of vials. TTP reflects the magnitude of bacterial load in blood and is often used as a prognostic marker by the clinicians to predict the clinical outcome.[6] Erroneous TTP may occur due to several factors such as delayed loading of vials and suboptimum blood volume. An erroneous TTP cannot be reliably used as prognostic marker.[7]

There have been very few studies that have examined the effect of DVL on organism recovery from blood culture vials and time to positivity.[4,5] To our best knowledge no such studies are available from India. More so, the automated blood culture (BACTEC and BACTALERT blood culture systems) has been recently introduced in our hospital. Therefore this study is designed with the purpose of evaluating the ability of two different continuously monitoring blood culture instruments (BACTEC and BACTALERT) to detect organisms from spiked blood cultures stored under a variety of temperatures for various lengths of time prior to loading into the instruments.

Materials and Methods
It is a prospective cohort study carried out in a tertiary care hospital in South India from January 2018 to April 2018. Two types of automated vials BACTALERT FA plus and BACTEC Plus Aerobic will be included in the study. Five recent clinical isolates of different microorganisms will be included in the study such as *Staphylococcus aureus, Streptococcus, Escherichia coli, Pseudomonas aeruginosa* and *Candida albicans*.

Inoculum preparation: For all except *C. albicans*, a suspension equivalent to a 0.5 McFarland standard (1.5

X 10^8 CFU/ml) will be prepared in sterile normal saline from 24- to 48-h fresh cultures. Then three subsequent 1:100 dilutions will be made in normal saline to obtain a final suspension of 1.5 X 10^2 CFU/ml. For *C. albicans*, a suspension equivalent to a 1.0 McFarland standard will be made in normal saline, and two subsequent 1:100 dilutions will be made to make a final suspension of 3.0 X 10^4 CFU/ml. From this final suspension, 0.3 ml will be mixed with 10 ml of anticoagulated human blood containing sodium citrate[8] (JIPMER Blood Bank, Pondicherry, India); which will be then inoculated into blood culture (BACTALERT FA plus and BACTEC Plus) vial to obtain a final inoculum of approximately 45 CFU per vial.

Vial loading: After inoculation, vials will be held for different holding time-temperature combinations as given in table-1. Vials will be incubated at three different temperatures 4°C, 37°C and room temperature [RT]. The different holding time will be 2, 6, 12, 24 and 36 h for each of the storage temperatures. To simulate the time spent (2h is usually acceptable) during two hours of holding time before loading into machines the vials incubated at 4°C and 37°C will be kept at RT. For e.g. vials held at 37°C for 36 h will be actually held at 37°C for 34 h and then 2h at RT. As up to 2 hours is usually considered as acceptable transport time therefore the 2 hour holding period will not be applicable for vials that will be incubated at 4°C and 37°C.

Thus, for an individual organism, there will be 13 temperatures- storage period combinations. Hence 13 BACTEC and 13 BACTALERT vials will be used for each organism as explained in the table 1. For each holding time- temperature combinations, one blood sterility control will also be used. Therefore, a total of 160 seeded vials and 26 control vials will be loaded in the instruments.

Table 1: Different holding time-temperature combinations used for delayed vial loading

		4°C	Room temperature		37°C	
	Bactec	**Bactalert**	**Bactec**	**Bactalert**	**Bactec**	**Bactalert**
2h	NA**	NA**	✓	✓	NA**	NA**
6h*	✓	✓	✓	✓	✓	✓
12h*	✓	✓	✓	✓	✓	✓
24h*	✓	✓	✓	✓	✓	✓
36h*	✓	✓	✓	✓	✓	✓

Note: *the last two hours incubated at room temperature ***NA, not applicable as 2hours is the recommended transport time. Hence no need for 2 hours incubation at 4°C and 37°C.

Organism recovery: All vials will be remained in the instruments for a maximum of 5 days unless they are flagged as positive by the instrument. Following a positive signal, TTP will be recorded, vials will be sub-cultured onto blood agar and MacConkey agar and then identification will be done by colony morphology & gram staining. All vials that do not give positive signal within the routine 5-day incubation period will be sub-cultured with 1 or 2 drops from each vial onto the blood agar and MacConkey agar. If growth occurs, then the culture vials will be declared as false negative. If growth does not occur, then those vials will be considered having non-viable organism and will be excluded from the study.

Statistical analysis

The categorical variables were expressed as frequency and percentages. The continuous variables were expressed either as mean with standard deviation or median with range. Differences in TTD and the numbers of false negative bottles were analyzed using nonparametric tests (the Mann-Whitney U test or the chi-square test where appropriate). A two-sided *P* value of <0.05 was considered statistically significant. All statistical analyses were performed with SPSS, version 16.0 for Windows (SPSS Inc., Chicago, IL).

Results

The test results were evaluated as per the description in Materials and Methods. Number of false-negative bottles for each temperature and time of incubation was noted as follows (Table 1)

Table 2 shows, the overall isolation rate of the pathogens was 96.92% and 90.7% in BACTEC and BACTALERT respectively. So out of total 130 inoculated bottles (except controls), 122 bottles flagged positive. 6 BACTALERT bottles and 2 BACTEC bottles gave false negative results. Maximum false negativity was obtained at 37˚C incubation temperature for 24 hours duration. The false-negativity increased with increase in the temperature of pre-incubation. There were 1, 2 and 5 false-negative results for bottles held at 4°C, RT & 37°C respectively. Also the number of false negatives increased with the increase in duration of incubation, i.e. after minimum 12 hours of incubation. Out of 8 false-negative bottles, 3 were shown by *Streptococcus pneumonia* & 2 by *Staphylococcus aureus* & 1 each by *E. coli* & *Pseudomonas.*

Table 2: Number of false-negative bottles for each temperature and time of incubation

Organism	Bottle type	No. of false-negative bottles for each temp (°C)/time (h) of pre-incubation													
		0	4°/6h	4°/12h	4°/24h	4°/36h	RT/2h	RT/6h	RT/12h	RT/24h	RT/36h	37°/6h	37°/12h	37°/24h	37°/36h
E. coli	Bactec	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Bactalert	-	-	-	-	-	-	-	-	-	-	-	-	`1	-
Pseudomonas	Bactec	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Bactalert	-	-	-	-	-	-	-	-	1	-	-	-	-	-
S. aureus	Bactec	-	-	-	-	-	-	-	-	-	-	-	-	-	1
	Bactalert	-	-	-	-	-	-	-	1	-	-	-	-	1	-
Streptococcus	Bactec	-	-	-	-	-	-	-	-	-	-	-	-	-	1
	Bactalert	-	-	-	-	-	-	-	-	-	-	-	-	1	-
Candida	Bactec	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Bactalert	-	-	1	-	-	-	-	-	-	-	-	-	-	-
Total	Bactec	0	0	0	0	0	0	0	0	0	0	0	0	0	2
	Bactalert	0	0	1	0	0	0	0	1	1	0	0	0	3	0

Table 3: Performance of BACTEC and BACTALERT (in terms of time to detection) based on varied holding time and temperature

Organism	***E .coli***		***Pseudomonas***		***S. aureus***		***Streptococcus***		***Candida***		**Average**	
Holding Temperature and duration	Bactec	Bactalert	Bactec	Bactalert	Bactec	Bactalert	Bactec	Bactalert	Bactec	Bactalert	Bactec	Bactalert
4°/6h	8.93	8.4	11	13.44	12.52	10.32	20.45	11.04	10.6	9.36	12.7	10.5
4°/12h	3.57	3.52	11.27	14.16	13.25	9.36	10.28	10.32	1.51	0	7.9	7.4
4°/24h	12.31	10.3	13.24	14.08	12.58	13.36	11.08	10.56	10.27	40.32	11.9	17.7
4°/36h	20.45	12.45	2.06	13.44	11.54	8.08	10.52	9.04	10.52	39.04	11	16.4
Average	11.3	8.7	9.4	13.8	12.5	10.3	13.1	10.2	8.2	22.2	10.9	13
RT/2h	7.76	8.02	11.27	12	11.34	10.08	0	9.36	8.59	8.32	7.8	9.6
RT /6h	4.06	9.2	5.64	12.72	8.45	10.6	7.48	9.6	6.06	13.44	6.3	11.1
RT /12h	8.91	9.2	4.3	9.12	7.87	0	6.52	5.52	7.75	7.12	7.1	6.2
RT /24h	1.32	4.21	2.85	0	3.34	4.56	3.07	4.32	3.26	25.44	2.8	7.7
RT /36h	1.96	2.04	0.68	3.6	1.01	2.32	1.01	10.32	20	18.16	4.9	7.3
Average	4.8	6.5	4.9	7.5	6.4	5.5	3.6	7.8	9.1	14.5	5.8	8.4
37°/6h	8.68	6.24	11.15	10.08	8.34	7.44	8.37	7.12	7.09	9.08	8.7	8
37°/12h	1.17	2.12	1.01	4.32	0.67	2.32	1.24	1.92	1.17	4.56	1.1	3.1
37°/24h	0.65	0	0.67	2	7.05	0	3.34	0	1.07	31.02	2.6	6.7
37°/36h	0.68	1.02	0.68	2.02	0	1.48	0	12	9.31	20.4	2.1	5.3
Average	2.8	2.3	3.4	4.6	4	2.8	3.2	5.2	4.7	16.2	3.6	5.8

As shown in table-3, in the present study we noted that best recovery with least time to detection was obtained when bottles were pre-incubated at 37°C for both BACTEC and BACTALERT i.e. average TTD for was 3.6hours and 5.8 hours for BACTEC and BACTALERT respectively. In BACTEC lowest average TTD were 7.9 hours, 2.8 hours and 1.1 hours at the holding time of 12hrs at 4°C, 24hrs at RT and 12hrs at 37°C respectively. Whereas in BACTALERT it was 7.4 hours, 6.2 hours and 3.1 hours at the holding time of 12hrs at 4°C, 36hrs at RT and 12hrs at 37°C respectively. We also noted that overall BACTEC had lesser time to detection compared to BACTALERT at all three holding temperatures (4°C, RT and 37°C). But statistical difference between the two systems was not significant(P-value>0.05).

Comparison the relative performance of the BACTEC and BACTALERT bottles showed that, the overall mean TTD for BACTEC bottles was 6.8 hours and for BACTALERT it was 9.1 hours. Organism wise analysis of TTDs showed that, the BACTEC bottles had average lesser TTDs for *Pseudomonas aeruginosa* (5.9 hours), *Streptococcus* (6.6 hours) and *candida albicans* (7.3 hours), whereas for *E. coli* (5.8 hours) and *S. aureus* (5.9 hours), whereas BACTALERT had lesser TTD.

Comparing the duration of incubation with TTD at various temperatures, earliest possible recovery of the organism was obtained from the bottles incubated at room temperature for 6 hours for both the instruments. So we established that storage of the inoculated bottles at room temperature gave the best recovery of the organisms for both BACTEC and BACTALERT.

Discussion

Bloodstream infections (BSIs) represent an important cause of human morbidity and mortality. The evaluation of patients suspected of having a BSI routinely includes blood cultures, which optimally yield an etiological diagnosis and provide the opportunity to perform antimicrobial susceptibility testing to guide therapeutic intervention when necessary.[9,10] Despite many improvements in medicine, blood cultures still remain the gold standard in microbiological diagnosis of blood stream infections.[11] Automated continuous-monitoring blood culture systems not only reduce the laboratory workload but they also accelerate the diagnosis.[12,13] The freshly inoculated blood culture bottles should be ideally transported to the laboratory and loaded into the continuous-monitoring instrument as soon as possible, in order to minimize the time to detection of microorganisms. However, because of off-site collection or restricted laboratory operating hours, there may be a substantial delay between blood culture inoculation and entry into the instrument. Several other factors are known to influence the time to positive detection (TTD) of the pathogens, such as inoculum size, delay in loading the bottles into the instrument, the incubation temperature, contamination, type of culture bottle, and the detection system used at the hospital.[14]

There is no such specific term called "delayed entry". But the term has been frequently used when the blood culture bottles are not loaded into the instruments timely. After loading of bottles TTD depends on the organism load, transport time to laboratory, volume of blood drawn (5-10ml ideally). So the delayed entry can affect the TTD effectively, which in turn will delay the isolation of organism and starting of appropriate treatment.[15] In our study, we have analyzed the effect of storage of the BACTEC & BACTALERT blood culture bottles at different pre-incubating temperatures (4℃, RT, 37℃) for different durations (2 hours, 12 hours, 24 hours, and 36 hours) on TTD under controlled conditions.

In our study, the isolation rates were 96.92% and 90.7% in BACTEC and BACTALERT respectively. So, BACTEC had superior sensitivity than BACTALERT system. These results corroborated findings in the published literature for comparisons of the BACTEC Plus Aerobic with the BACTALERT FA.[16,17] Maximum false-negative results were obtained at 37°C for both BACTEC & BACTALERT bottles which was in concordance with the results by Lemming *et al* who reported high false-negative rate for BACTEC bottles at 35°C.[18] We got the best recovery of the organism with least TTD with BACTEC at pre-incubation temperature of 37 ℃. While Lemming *et al* in 2006 in their study found a least TTD with pre-incubation at 35°C and they obtained best recovery from bottles held at 4°C.[18] Our study results are in concordance with the study by Eon-Ha Kohl *et al* done in 2013, which showed that 37℃ pre-incubation was advantageous over RT in detection of organisms in blood culture systems, when delayed entry is inevitable.[19] BACTEC bottles detected *Pseudomonas aeruginosa*, *Streptococcus* and *candida albicans* earlier whereas for *E. coli* and *S. aureus* were detected faster by BACTALERT, whereas a study done by Cockerell *et al* showed early detection of *Enterobacteriaceae*, *Pseudomonas* spp, *S.aureus* and *Candida* species by BACTEC.[20]

BACTEC showed the lowest average TTD at the holding time of 12hrs at 4°C, 24hrs at RT and 12hrs at 37°C respectively. Whereas, in BACTALERT lowest average TTD was obtained at the holding time of 12hrs at 4°C, 36hrs at RT and 12hrs at 37°C respectively. Hence TTD showed a decreasing trend with increasing duration of pre-incubation. This was in concordance with the study done by Lemming *et al* who found in their study that, TTD was inversely influenced by the holding time. In the study, we found that BACTEC detects lesser TTD than BACTALERT machine at all the three temperatures i.e. at 4℃, room temperature and 37℃,whereas in the study done by Lemming *et al.*[18] Average mean TTD was 31.1 h for BACTEC bottles and 32.8 h for BACTALERT bottles. So BACTEC is

comparatively more efficient than BACTALERT according to both the studies.[18,21]

There are no such recommendations depicting delayed entry of blood culture bottles. The *Manual of Clinical Microbiology* (11th edition) recommends that the blood culture bottles can be stored for up to 2 hours unless otherwise specified by the manufacturer.[22] The package insert of BACTALERT blood culture bottles recommend that bottles be loaded immediately after inoculation. But a recent memo by BioMe´rieux suggested that inoculated bottles can be held at room temperature if delayed entry is inevitable.[23] Instruction manual by BD Diagnostics suggests that BACTEC bottles can be held for up to 20 h at incubator temperature (temperature not specified) or up to 48 hours at RT.

Reductions in the numbers of technical personnel in the peripheral laboratory and hours of operation, as well as off-site specimen collection, are becoming more common. Often, these measures result in delayed entry of blood culture bottles into instruments. Since the detection algorithms of continuously monitoring instruments are based on significant changes in microbial growth characteristics, multiple factors regarding these systems and delayed entry of bottles need to be addressed. These include the optimal pre-incubation temperature, the maximum time that a bottle can be delayed outside of the system, and the necessity of performing entry and/or terminal subcultures.[24]

In our study, we concluded that BACTEC bottles give a better recovery of organisms that BACTALERT at all the incubation temperatures i.e. 4˚C, RT and 37˚C. TTD wise analysis gave best recovery at 37˚C by both the systems which is in concordance with the results shown by Velden *et al.*[25] However higher occurrence of false-negative results after pre-incubation at 37°C for at least 24 h than after storage at room temperature.[5,25] We noted an inverse relationship between storage time and temperature. But duration wise analysis showed that, pre-incubating both BACTEC and BACTALERT bottles at room temperature can be beneficial for receiving the earlier final reports when delayed entry is inevitable. It will help in storing the blood culture bottles in the peripheral set-ups where round the clock laboratory facility is not available .But our conclusions are based upon the data generated from blood culture bottles inoculated with 5 (*E. coli, Pseudomonas, S. aureus, Streptococcus, Candida*) known microorganisms under controlled conditions. Further studies may be conducted for other organisms and the relationship between the delayed entry and time to detection can be studied.

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