Evaluation of direct light microscopy for rapid detection of microorganisms in blood cultures

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ABSTRACT

Objectives: The aim of this study was to evaluate the use of direct light microscopy (x 40 objective) of unstained uncentrifuged blood-broth mixtures for rapid detection of microorganisms in blood cultures.

Methods: Two thousand two hundred and ninety-four blood cultures were investigated in this study. Blood cultures were processed in the Clinical Microbiology Laboratory, Queen Alia General Hospital, Amman, Jordan from January to December 2001. We compared the results of direct light microscopy and subcultures in 3 stages: macroscopically positive blood culture bottles, macroscopically negative blood culture bottles after 9-17 hours, and macroscopically negative blood culture bottles after 7 days of incubation.

Results: The total positive blood cultures was 434 (18.9%). Three hundred and fifty three (81.3%) were positive by macroscopic examination and direct light microscopy and grew viable organisms, 34 were macroscopically negative blood cultures that grew viable organisms after 9-17 hours of incubation in which 32 (94.1%) were positive by direct light microscopy and 47 macroscopically negative blood cultures that grew viable organisms after 7 days of incubation in which 45 (95.7%) were positive by direct light microscopy.

Conclusion: The direct light microscopy methodology proved to be simple, rapid, cost effective, accurate and sensitive technique for the early detection of bacteremia.

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In large studies, the mortality rate of the bacteremia ranges between 20-50%.[1-4] Therefore, prompt isolation and accurate identification of the etiological agents remain among the most important functions performed by the Clinical Microbiology Laboratory. Conventionally, the detection of microorganisms in blood cultures relies mainly upon daily macroscopic inspection of undisturbed supernatant broths for any visible evidence of bacterial growth (for example, turbidity, hemolysis, gas production, chocolatization of the blood, and the presence of visible colonies or a layer of growth on the sedimented blood layer). A number of automated blood culture systems have been developed as an automated substitute for daily visual inspection allowing for more efficient and earlier detection of microbial growth. Older automated systems such as Becton Dickinson Microbiology Systems (BACTEC) radiometric and non-radiometric systems are no longer in use. Continuous-monitoring blood culture systems (CMBCS) have been the most important technological advance in blood cultures during the past 20 years. Continuous-monitoring blood culture systems have been previously described in detail[5] and updated information has been published recently.[6] Considerable research on the use

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of nucleic acid probes with or without amplification for the identification of infectious agents is in progress. However, none of these is available for routine use for the detection of bacteria and fungi in blood culture bottles or directly from the blood, they search an alternative rapid methods for the future.[7] In the Microbiology Laboratory of Queen Alia General Hospital, Amman, Jordan, the direct light microscopy (x 40 objective) of the unstained uncentrifuged blood-broth mixtures is a routine technique that was performed as part of the adopted conventional broth blood culture system. The purpose of this study was to evaluate the sensitivity, specificity, and predictive values of the direct light microscopy for the detection of microorganisms in blood cultures.

Methods. Blood cultures studied were processed in the Clinical Microbiology Laboratory at the Queen Alia General Hospital, Amman, Jordan for the 12-month period of 2001. Approximately 15-20 ml of blood was aseptically drawn from adult patients, and equal samples were inoculated into each of 2 70-ml bottles containing Columbia broth (Becton Dickinson, Sparks, Maryland) with sodium polyanethol sulphonate (SPS) (Liquoid) under vacuum with carbon dioxide (CO_2). Approximately 3-5 ml of blood was aseptically drawn from pediatric patients and inoculated into one 20-ml Columbia broth bottle. Upon arrival in the laboratory, one bottle (set of 2) collected from the same adult patient was vented to admit air, while all other blood culture bottles remained unvented. All bottles were incubated at 35^{0}C up to 7 days or until microbial growth was detected. The undisturbed supernatant broths were inspected daily for any macroscopic evidence of microbial growth. Direct light microscopy (x 40 objective) of the unstained uncentrifuged blood-broth mixtures was performed: 1) immediately on all macroscopically positive blood culture bottles, 2) after 9-17 hours on all macroscopically negative blood culture bottles and 3) after 7 days on all macroscopically negative blood culture bottles. Subcultures, direct biochemical tests, and direct antimicrobial susceptibility testing were performed according to the organism present in all microscopically positive blood culture bottles. Blind early and terminal subcultures into blood agars were performed after 9-17 hours and 7 days of incubation for all macroscopically negative blood culture bottles. All subcultures were incubated at 35^{0}C in 5% CO_2. Plates were held for 48 hours and inspected for evidence of growth at 12-hour intervals.

Reporting of the results. The morphology and arrangement of the organisms seen (for example, cocci in clusters, chains, pairs or tetrads, coccobacilli, *bacilli* in long chains, budding yeast and so forth) were reported immediately either by telephone or personal ward visits to determine the clinical relevance of the organisms and to provide the necessary antibiotic therapy.

Statistical methods. The results of direct light microscopy were studied in comparison with isolation of viable organisms in subcultures. The sensitivity, specificity, and predictive values were calculated by standard formulas. A true positive culture was defined as a positive smear from a culture bottle that subsequently grew an organism within 48 hours. A false positive culture has a positive smear but has a negative culture within 48 hours. A false negative culture has a negative smear but has a positive culture within 48 hours of incubation.

Results. A total of 2,294 blood culture bottles were included in this study. Of 434 (18.9%) positive culture bottles from 387 patients, 430 (99.1%) were positive by direct light microscopy. Of 376 macroscopically positive blood culture bottles, 353 (81.3%) were positive by subculture and grew viable organisms. There were 207 (58.6%) *Staphylococcus epidermidis (Staph. epidermidis)* followed by 44 (12.5%) *Klebsiella species* 28 (7.9%) *Polymicrobial organisms* and 24 (6.8%) *Escherichia coli* (*E. coli*). The 23 (6.1%) macroscopically positive blood culture bottles, which were negative by direct light microscopy and did not grow viable organisms, were taken from newborns. There was a total agreement between direct light microscopy and subculture results of blood cultures at first stage. Of 1,941 macroscopically negative blood culture bottles after 9-17 hours of incubation, 34 (7.8%) were positive by subculture and grew viable organisms. There were 10 (29.4%) polymicrobial followed by 6 (17.6%) Gram positive anaerobes, 4 (11.8%) *E. coli* and 3 (8.8%) *Staph. epidermidis*. Direct light microscopy showed 32 (94.1%) true positives, 3 (8.8%) false positives and 2 (5.9%) false negatives (**Table 1**). Therefore, the sensitivity of direct light microscopy at 2nd stage was 94.1%, specificity was 99.8%, positive predictive value was 91.4% and negative predictive value was 99.9%. Of 1,907 macroscopically negative blood culture bottles after 7 days of incubation, 47 (10.8%) were positive by subculture and grew viable organisms. There were 13 (27.7%) *Staph. epidermidis* followed by 7 (14.9%) *Candida species*. and 6 (12.8%) *Pseudomonas species*. Direct light microscopy showed 45 (95.7%) true positives, 5 (10.6%) false positives and 2 (4.3%) false negatives (**Table 2**). Therefore, the sensitivity of direct light microscopy at 3rd stage was 95.7%, specificity was 99.7%, positive predictive value was 90.0% and negative predictive value was 99.9%. **Table 3** showed the organisms and their time of detection. It was noted that *Pseudomonas species*. (66.7%) and

Table 1

Results	Positive microscopy	Negative microscopy	Total
Positive subculture	32	2	**34**
Negative subculture	3	1904	**1907**
Total	**35**	**1906**	**1941**

n - number

Table 1 - Results of direct light microscopy and blind early subculture after 9-17 hours of incubation of macroscopically negative blood cultures (n=1941).

Table 2

Results	Positive microscopy	Negative microscopy	Total
Positive subculture	45	2	**47**
Negative subculture	5	1855	**1860**
Total	**50**	**1857**	**1907**

n - number

Table 2 - Results of direct light microscopy and terminal subculture after 7 days of incubation of macroscopically negative blood cultures (n=1907).

Table 3 - Organisms isolated and time of detection.

Table 3

Organism	Early detection	9-17 hours after incubation	7 days after incubation	Total
Escherichia coli	24	4	3	**31**
Klebsiella sp.	44	2	2	**48**
Enterobacter sp.	4	1	0	**5**
Pseudomonas sp.	2	1	6	**9**
Serratia marcescens	1	0	0	**1**
Acinetobacter sp.	1	0	0	**1**
Proteus sp.	1	0	0	**1**
Salmonella sp.	4	1	1	**6**
Gram-negative rods, miscellaneous	3	0	4	**7**
Bacteroides sp.	2	1	1	**4**
Staphylococcus aureus	9	2	2	**13**
Staphylococcus epidermidis	207	3	13	**223**
Streptococcus pyogens	1	0	0	**1**
Streptococcus agalactiae	1	0	0	**1**
Enterococcus sp.	1	0	0	**1**
Streptococcus pneumoniae	2	1	0	**3**
Streptococcus viridans	2	0	0	**2**
Gram positive cocci, miscellaneous	7	1	1	**9**
Listeria monocytogenes	1	0	0	**1**
Bacillus sp.	1	0	0	**1**
Corynebacterium sp.	1	1	1	**3**
Gram positive anaerobes	6	6	2	**14**
Polymicrobial	28	10	4	**42**
Candida sp.	0	0	7	**7**
Total	**353**	**34**	**47**	**434**

sp - species

Candida species. (100%) did not show early macroscopic evidence of growth and grew after 5 days of incubation.

Discussion. Although both the conventional subculture and direct light microscopy methods appear to be equivalent, subcultures require 24-48 hours incubation period, while direct light microscopy allows the same day detection of microorganisms with clear advantage of earlier detection, identification, and antimicrobial sensitivity testing. The 23 macroscopically positive blood culture bottles, which were microscopically negative and did not grow viable organisms, were from newborns. This might be due to the relatively high white cell count of the newborns associated with increased metabolic activity and resulting in macroscopically suspicious looking blood culture bottles. The 8 false positive results detected by direct light microscopy were attributed to the presence of aggregated platelets mimicking cocci in clusters or the accidental use of unclean glass slide, cover slide or microscope lens. It has been previously reported[8] that false positive results were due to the detection of non-viable organisms representing either procedural contaminants or non-viable bacteria in the blood culture medium introduced during the preparation of the culture bottles by the manufacturer. Most of the non-viable organisms were recognized for their morphology and variable staining characteristics. The 4 false negative results detected by direct light microscopy were likely related to the size of the inoculum and the duration of incubation where the presence of at least 10 organisms/ml was required for possible detection.[8,9] Many clinical microbiology laboratories use the Gram, methylene blue or acridine orange stained smears for initial microscopic examination of macroscopically negative blood cultures within 24 hours of receipt. The sensitivity of the Gram stain has been documented.[10,11] However, the direct microscopic examination of blood cultures by Gram or methylene blue stain at x 1,000 magnification is tedious, time consuming and frequently insensitive.[10] The use of acridine orange stain for the detection of microorganisms in blood cultures has also been assessed,[12] it allows scanning at x 600 magnification and confirming with oil immersion, thus speeding up the task with greater sensitivity than the Gram stain.[8,10] Since macroscopic evidence of microbial growth may be difficult to detect after 2-3 days of incubation due to hemolysis or absence of hemolysis, blood culture bottles which were processed with manual blood culture systems should be incubated for 7 days with terminal subcultures. It has to be emphasized that while a total of 7 days of incubation of blood cultures is probably adequate for general hospitals, the 2nd week of incubation should be indicated in selected cases of suspected endocarditis and persistent or recurrent infection, as well as in any referral center. The group of fastidious gram-negative bacilli that includes *Hemophilus aphrophilus, Actinobacillus actinomycetemcomitans, Cardiobacterium hominis, Eikenella corrodens* and *Kingella kingae* (HACEK), usually requires extended incubation for their detection.[13] It was noted that organisms like *Pseudomonas* and *Candida species* frequently did not produce turbidity in blood culture broths and they probably would not have been detected until the terminal subculture was performed. It has been reported that *Pseudomonas aeruginosa* and pathogenic yeasts are strictly aerobic and are unlikely to grow to detectable levels in blood culture bottles containing insufficient oxygen.[14-18] Some organisms fail to produce visible evidence of growth and others grow slowly resulting in delayed detection. It was recommended to use either subcultures or Gram stains of macroscopically negative blood culture bottles after one or 2-days incubation period and again before a final negative culture result after 7 or 14 days of incubation.[19-21] The value of Gram staining and early subculture for the initial detection of positive blood cultures has been demonstrated.[8,10,16,20-22] However, the optimal time for performance of routine Gram stains and subcultures has varied drastically.[16,21,23] The value of an early Gram stain (7-19 hours after collection) and of inspection of early subculture plates for growth after 12 hours of incubation has also been studied.[10] A comparative study evaluating the use of subculture, Gram stain, and macroscopic methods indicated that Gram stain and subculture first detected the presence of microorganisms in 23 and 12% of positive cultures.[20] The recovery of an organism from bloodstream should always be considered significant until proven otherwise. Previous studies[24,25] showed that *Staph. epidermidis* are the most frequently encountered coagulase negative species in blood cultures. Similarly, *Staph. epidermidis* had the highest isolation rate (51.4%) in our study. However, more than 94% of these organisms, as compared to 85% in previous studies,[26] were judged to be contaminants after discussions with the clinicians and in the light of clinical picture of the patients. Every clinical microbiology laboratory needs policies and procedures designed to ensure that blood cultures are collected in such a way as to minimize contamination. Whether blood cultures are collected by medical technologists, trained phlebotobists, nurses, or other health care workers, an ongoing program to monitor compliance with these policies and procedures is necessary and should be part of the laboratory quality assurance program. Guidelines for proper blood cultures collection and reduction of contamination have been recently published.[27]

Based on our results, we concluded that direct light microscopy of the unstained uncentrifuged blood-

broth mixtures is simple, rapid, cost-effective, accurate and sensitive technique for early detection of bacteremia. It is recommended for laboratories that process a small numbers of blood cultures. In the referral centers with larger numbers of specimens, such technique could be useful in the routine examination of macroscopically positive blood culture bottles; however, it should not be a substitute to the new automated blood culture systems if affordable. The techniques involved in the detection of microorganisms in the bloodstream have steadily improved with the introduction of CMBCS. These systems contribute a better and early detection of microbial growth in addition to decreased laboratory workload. However, the cost, the limited medium types of some systems, and the size of certain instruments are the main disadvantages.[6]

Further studies are required to assess the use of direct light microscopy in examination of blood culture bottles of the automated systems.

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