RESEARCH LETTER

Fluorescent in situ hybridization and Gram-stained smears of whole blood as complementary screening tools in the diagnosis of sepsis

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Introduction According to the Sepsis Definitions Task Force of the Society of Critical Care Medicine and European Society of Intensive Care Medicine, sepsis is currently defined as "life-threatening organ dysfunction caused by a dysregulated host response to infection,"[1] and its diagnostic criteria rely on sepsis-related organ failure assessment scores—SOFA or qSOFA. Sepsis is diagnosed when SOFA score is 2 points or higher, if infection is present or suspected, or if there are 2 or more symptoms according to the qSOFA score (disorders of consciousness, systolic blood pressure ≤100 mmHg, respiratory rate ≥22 breaths/min). Additionally, systemic inflammatory response syndrome was left out of the definition of sepsis and the term "sepsis" replaced "severe sepsis."

A proinflammatory response developing in an uncontrolled way in the body can lead to multiple organ dysfunction syndrome, including death. Therefore, discovering the source of infection early and determining the etiologic agent are crucial for the treatment optimization, employing effective targeted antibiotic therapy, and consequently, improving the prognosis for patients with sepsis.

Detection of bacteria in blood might prove difficult owing to their relatively small number and periodic seeding into the bloodstream, and also as a result of the antibiotic therapy applied, which reduces the chance of culturing them. Currently, the gold standard in microbiological diagnostics of blood is its culture in automated systems (eg, BACTEC [BectonDickinson]). The disadvantage is that this method is time-consuming, taking even several days, and has low sensitivity, which allows to detect microbial growth only in 15% to 30% of the culture.[2]

To improve the effectiveness of identifying the etiological agent in blood, attempts have been undertaken to detect lipopolysaccharide for Gram-negative bacteria or mannan and galactomannan for fungi, using serological methods. Other promising diagnostic tools are significantly faster and more precise molecular methods based on polymerase chain reaction (PCR). Molecular biology techniques are independent of prior antibiotic treatment and do not require bacterial or fungal growth in a culture medium.[3,4]

Serological or molecular diagnosis based on the PCR methods is costly and requires specialized diagnostic laboratories. Screening methods would be extremely valuable as they could quickly reveal the presence of bacteria in the direct smear. Such methods include Gram staining and fluorescent in situ hybridization (FISH). Therefore, the objective of this study was to compare culturing with the modified method for blood sample preparation for Gram staining and FISH in the direct smear from blood.

Patients and methods A total of 53 blood samples from adult patients with suspected sepsis were analyzed on the basis of the clinical picture and laboratory tests results. The blood samples were taken from patients of the Department of Anesthesiology and Intensive Care in the John Paul II Hospital in Kraków, Poland, in the years from 2012 to 2013. The research was approved

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Received: December 12, 2016.
Revision accepted: January 20, 2017.
Published online: February 28, 2017.
Conflict of interests: none declared.
Pol Arch Intern Med. 2017; 127 (2): 122-124
doi:10.20452/pamw.3949

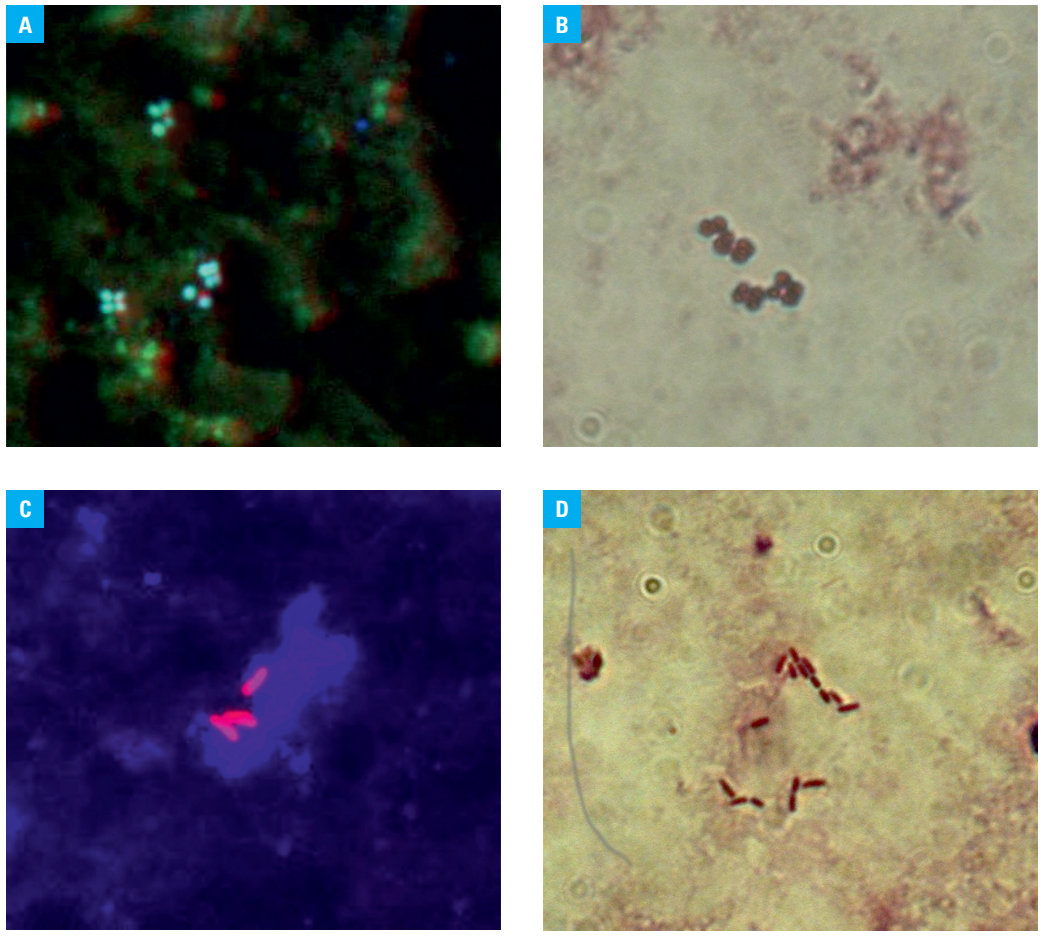

FIGURE 1 Microscopic images (zoom, ×1000); **A** – fluorescent in situ hybridization (FISH) smear with STA and EUB338 probes: visible *Staphylococcus* spp cells; **B** – Gram smear: visible Gram-positive cocci; **C** – FISH smear with ENT183 and EUB338 probes: visible *Enterobacteriaceae* cells; **D** – Gram smear: visible Gram-negative bacilli

by the Jagiellonian University Bioethics Committee (KBET/94/B/2009). Blood was drawn from patients who met the existing clinical criteria for sepsis.

The blood culture was carried out in the John Paul II Hospital in the Department of Microbiology, using the BacT/ALERT® 3D apparatus (bioMérieux, Hazelwood, Missouri, United States). Simultaneously, the blood samples were analyzed using FISH and Gram staining.

Fluorescent in situ hybridization in whole blood Blood samples were subjected to in situ hybridization according to the method described by Gosiewski et al.[5] In order to detect bacteria, the following fluorophore-labeled probes (Genomed, Warsaw, Poland) were used: probe EUB338 to detect all species of bacteria cumulatively: (FITC-5'– GCT GCC TCC CGT AGG AGT– 3'-FITC)[6]; probe STA—for the genus *Staphylococcus*: (CY3-5'– TCC TCC ATA TCT CTG CGC 3')[7]; and probe ENT183—for *Enterobacteriaceae* (CY3-5'- CTC TTT GGT CTT GCG ACG-3').[8]

The preparations were analyzed using the BX51 fluorescence microscope (Olympus, Ontario, Canada) equipped with an immersion objective 100×, an ultravioloet lamp, and an F-View camera (Olympus). Image analysis was carried out using the AnalySIS software (Soft Imaging, Ontario, Canada).

Preparation and gram staining of direct blood smears Direct smears were prepared from blood samples ready for determination using FISH by conducting hemolysis of erythrocytes with a solution of ammonium chloride, and the leukocyte precipitate obtained was used to prepare microscope slides.[5] Subsequently, standard Gram staining was applied. The preparations were examined using the CX21 light microscope (Olympus).

Statistical methods For statistical analysis, the 2-tailed Fisher exact test was used (Gretl software ver. 1.9.4., Toruń, Poland).

Results In total, 53 blood samples were tested using the blood culture, Gram-stained direct smears, and FISH (FIGURE 1A–1D), and the following percentages of positive results were obtained: 26.4% (n = 14); 43.4% (n = 23), and 50.9% (n = 27), respectively. Sensitivity and specificity for Gram staining and FISH compared to culture was 85.7% and 71.8% as well as 100% and 66.7%, respectively. Significant differences were found between the results obtained through blood culture and Gram staining ($F = 0.00032$; $P < 0.01$) as well as culture and FISH ($F = 0.00012$; $P < 0.01$). The time required to obtain test results using Gram staining and FISH amounted to about 4 to 5 hours, while for the culture it was even 72 hours.

Discussion Despite the tremendous progress in the diagnosis and treatment of sepsis, blood

culture still remains the gold standard in everyday clinical practice. Undoubtedly, the merits of this test are its simplicity, low cost, and the possibility of determining bacterial susceptibility to antibiotics. The downside of bacterial culture in blood is the time required to perform the test and low sensitivity. Bacteria can be cultured from blood only in about 15% to 30% of cases, and the result is obtained after 3 to 5 days, which may have serious consequences for the patient's health or even life.[5,9] Hence, studies are being conducted to find alternative diagnostic methods, and they are mainly based on molecular biology research.[2,4] Recently, our team demonstrated the presence of bacterial DNA in the blood of both healthy individuals and patients with sepsis. We noticed considerable taxonomic diversity between the 2 groups. Among healthy individuals, anaerobic bacteria DNA dominated in the blood, and in the group with sepsis, aerobic and microaerophilic bacteria DNA was mainly present.[10] It brings us to a conclusion that the blood from each patient with symptoms of sepsis should be routinely tested for bacteremia.

In our study, we employed the FISH technique and Gram staining for direct smears of patients' blood subjected to preliminary preparation. It is common knowledge that Gram-stained direct smears do not constitute valuable diagnostic material because the image is obscured by erythrocytes. In literature, numerous reports can be found on the use of FISH for testing samples of fluids following blood culture.[11,12]

Owing to hemoglobin sample purification in accordance with the methodology described by Gosiewski et al,[5] in which a clear microscopic picture of good quality was obtained without background autofluorescence, the FISH method can be successfully applied today for whole blood, without prior culture. Analogous methods were used in the preparation of Gram smears, wherein erythrocytes were initially removed, and at the same time, the sample was concentrated.

High sensitivity of FISH testing (100%) and Gram staining (85.7%) was observed, but specificity was 85.7% and 71.8%, respectively. This difference most probably stems from the fact that a blood culture reveals only living and proliferating bacteria, while FISH and Gram staining can also confirm the presence of dead cells.[12] Blood culture has numerous limitations, including a small number of microbes in the blood and the use of antibiotics that inhibit the growth of bacteria. Furthermore, the strains cultured could have started through contamination. The shortcoming of our study was that we did not monitor the time of blood collection for microbiological testing in relation to the applied antibiotic treatment.

The highest proportion of positive results was obtained using the FISH method. Therefore, it seems that this method, based on molecular biology, may be successfully applied in laboratory diagnosis of sepsis as a screening tool while waiting for the results of standard laboratory tests. The applied Gram staining may also be considered a rapid screening test on similar principles as cerebrospinal fluid direct smear testing; however, it shows lower sensitivity and specificity than FISH.

Excluding the fluorescence microscope and thermocycler equipment, the cost of FISH and Gram-staining analysis is lower than that of the PCR amplification.

The search for alternatives to the gold standard of blood culture is still a pressing issue. The methods that we propose could be used at least as complementary diagnostic tools before further progress in the diagnosis, management, and care of patients with sepsis is made.[13]

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