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Application of Fluorescent *In Situ* Hybridization for Quick Identification of Microorganisms from Positive Blood Cultures

Gergana Lengerova[1,2,3]*, Yordan Kalchev[1,2,3], Zoya Rachkovska[1,2,3], Ralitsa Raycheva[4], Michael Petrov[1,3], Marianna Murdjeva[1,2,3]

[1]Department of Microbiology and Immunology, Faculty of Pharmacy, Medical University, Plovdiv, Bulgaria; [2]Laboratory of Microbiology, University Hospital St. George, Plovdiv, Bulgaria; [3]Research Institute, Medical University, Plovdiv, Bulgaria; [4]Department of Social Medicine and Public Health, Faculty of Public Health, Medical University, Plovdiv, Bulgaria

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***Correspondence:** Gergana Lengerova, Department of Microbiology and Immunology, Medical University of Plovdiv, 15-A Vasil Aprilov Blvd., 4002, Plovdiv, Bulgaria. E-mail: gergana.lengerova@mu-plovdiv.bg
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Abstract

AIM: The aim of this study was to evaluate the diagnostic potential of the fluorescent *in situ* hybridization (FISH) method for quick identification of microorganisms from positive blood cultures.

MATERIALS AND METHODS: QuickFISH BC is a multicolor, qualitative nucleic acid hybridization assay using specific fluorescent-labeled probes for identification of Gram-positive bacteria (*S. aureus,* Coagulase-negative *Staphylococcus spp*. – CoNS*, E. faecalis,* and *E. faecium*); Gram-negative bacteria (*E. coli, P. aeruginosa,* and *K. pneumoniae*), and fungi (*C. albicans, C. tropicalis*, and *C. glabrata).* This method applied to 72 positive blood cultures obtained from patients admitted at the University Hospital St. George – Plovdiv. A preliminary selection based on Gram staining was performed before the application of the FISH test. All microorganisms were subject to identification by routine biochemical tests, semi-automated and automated systems as well. Statistical data processing included descriptive statistics, nonparametric analysis for testing hypotheses by SPSS v. 22.0, and Microsoft Excel software. $p < 0.05$ was considered statistically significant.

RESULTS: FISH detected microorganisms in 63 (87.5%) positive blood cultures, whereas no fluorescent signal was observed in 9 (12.5%). The latter was because not all the microorganisms we identified are included in the test spectrum, for example – *Enterobacter spp.* and *Acinetobacter spp.* By FISH, we found *S. aureus* in 10 (15.9%) cases, CoNS in 20 (31.6%), *E. faecalis* in 4 (6.4%), and *E. faecium* in 4 (6.4%). *E. coli* (n = 7; 11.1%) was the leading cause of bacteremia among Gram-negative bacteria, whereas *C. albicans* predominated (n = 4; 6.4%) among fungi.

CONCLUSION: QuickFISH BC is a rapid and accurate screening method for the identification of some of the most frequent pathogens causing bacteremia. This enables the initiation of the early and adequate antimicrobial therapy. The lack of pathogen identification from positive blood cultures using this method implies the need to continue identification with other tests.

Introduction

The presence and circulation of pathogenic microorganisms in the bloodstream can lead to severe life-threatening conditions such as sepsis – the most serious infectious complication. The rapid diagnosis and initiation of effective antimicrobial therapy are an important factor in its management. The causative agents of bloodstream infections (BSIs) can be various microorganisms – most often bacteria and fungi, while viruses are significantly rarer. This etiological diversity complicates the diagnosis and the choice of antimicrobial treatment, as it depends on the specific causative agent. The severity of BSI and their growing prevalence caused by multidrug-resistant bacteria and fungi poses a serious threat to public health worldwide [1], [2], [3]. Each year over 1,700,000 cases of BSIs are diagnosed in Europe and North America, responsible for over 230,000 deaths per year, resulting in a mortality rate between 10 and 40% [4], [5], [6], [7], [8]. Bloodstream infections and related septic conditions are a major problem in industrialized countries [9], [10].

The modern approach to treatment requires the initiation of the early empirical therapy. Its delay is associated with an unfavorable outcome for the patient. Several studies highlight the fact that the use of appropriate antibiotics and the quick detection of the causative agent reduces mortality from BSIs by 30% [4], [5], [6], [7], [8].

The classical microbiological examination is relatively slow (1−7 days), depending on the growth and replication of the microorganisms in the culture medium and their initial concentration inside the blood culture. Fast and more adequate methods for the identification of microorganisms still need to be fully validated. Standard microbiological tests for the identification of microorganisms require at least 48−72 h to a result after the blood culture has become positive. In comparison, quick diagnostic tests may provide definitive identification of the microorganism

within hours [11], [12], [13], [14]. To reduce the time to identification of pathogens in positive blood cultures, a variety of modern techniques are used – biochemical analysis of isolates, radiometric systems, *in situ* hybridization, mass spectrometry, and molecular genetic methods of diagnosis. These methods are very promising due to their high sensitivity.

Peptide nucleic acids (PNA), which are pseudo-peptides, serve as the basis of fluorescent *in situ* hybridization (FISH). The Watson and Crick complementarity rules fully apply to them. PNA probes have suitable characteristics such as high specificity, affinity, and fast kinetics. This results in an improved hybridization to highly structured targets – rRNA [15], [16]. Direct hybridization of positive blood cultures with probes in the fluorescent *in situ* hybridization technique is limited to the identification of one or several specific bacterial species [17], [18]. This protocol provides direct identification of the microbial agent in 25−45 min, depending on whether Gram-positive, Gram-negative bacteria, or yeast-like fungi, are present [19]. The following study aims to determine the diagnostic capabilities of the FISH method for the rapid identification of microorganisms from positive blood cultures.

Materials and Methods

Materials

We examined 72 positive blood cultures from patients hospitalized at the clinics of the University Hospital St. George – Plovdiv. The specimens were collected through venipuncture in compliance with the antiseptics procedures from patients with clinical and laboratory abnormalities consistent with a bloodstream infection. The blood cultures were transported as soon as possible to the Laboratory of Microbiology at the University Hospital St. George – Plovdiv and to the Department of Microbiology and Immunology of the Medical University – Plovdiv. The blood culture bottles were incubated in BacT/ALERT 3D-60 system (bioMérieux, France). This is an automated system for incubation and detection of microbial growth in blood cultures bottles. When the BacT/ALERT system recognized a positive blood culture, the process continued with the preparation of microscopic slides stained by gram and application of FISH by QuickFISH BC test (AdvanDx, Woburn, MA), followed by a standard microbiological examination and identification.

Methods

QuickFISH BC

Multicolor qualitative test for fluorescent *in situ* hybridization of nucleic acid by specific probes for the detection of Gram-positive bacteria *(S. aureus,* Coagulase-negative *staphylococci* (CoNS)*, E. faecalis,* and *E. faecium*), Gram-negative bacteria (*E. coli, P. aeruginosa,* and *K. pneumoniae*), and fungi (*C. albicans, C. parapsilosis,* and *C. glabrata)*. The PNA FISH method of AdvanDx was introduced into the Laboratory of Microbiology of the University Hospital St. George – Plovdiv in 2015. After the routine incubation of blood cultures by automated system BacT/ALERT 3D-60 (bioMérieux, France), phenotypic preliminary identification of positive samples (blood cultures) was performed by Gram stain. Depending on the used test for Gram-negative bacteria, *Staphylococcus spp., Enterococcus spp.,* or *Candida spp*., different organisms provided distinct color fluorescence – yellow, red, and green. The test started immediately after the establishment of the presence of a microorganism in the Gram stain. 100−150 μl of blood was added to an AdvanDx microtube with an antibiotic removal filter using the neutralizing ion exchange resins contained in the blood culture media. Ten microliters of the thus treated sample were transferred to the center of an AdvanDx slide, previously set on a thermal cycler at 55°C ± 1°C. The samples were fixed with two solutions (Quick Fix 1 and Quick Fix 2). After the fixation of the sample on a slide – one drop of PNA blue and one drop of PNA yellow were mixed (containing fluorescein-labeled probes for 16S rRNA sequences specific to the species, respectively). The two reagents were mixed until uniformly green in color. The next step involved placing the cover glass on the slide and hybridizing at 55°C ± 1°C for 15 min. The samples were observed at ×100 magnification immersion objective of a fluorescent microscope (Nikon Eclipse 80i). QuickFISH slides had built-in positive and negative controls, which were reported together with the sample. In the positive control of each of the listed species, there was a different color of fluorescence. The spectrum of the test and the color of the fluorescence are shown in Table 1. Negative (without microorganisms) controls do not give fluorescence.

Table 1: Possibilities for identification of microorganisms by Quick fluorescent *in situ* hybridization BC test

Microorganism	Type of fluorescence
Gram-positive bacteria	
Enterococcus faecalis	Green fluorescence
Enterococcus faecium	Red fluorescence
Staphylococcus aureus	Green fluorescence
CoNS	Red fluorescence
Gram-negative bacteria	
Escherichia coli	Green fluorescence
Pseudomonas aeruginosa	Red fluorescence
Klebsiella pneumoniae	Yellow fluorescence
Fungi	
Candida albicans	Green fluorescence
Candida glabrata	Yellow fluorescence
Candida tropicalis	Red fluorescence

CoNS: Coagulase-negative *staphylococci.*

Routine microbiological examination

Selective and non-selective culture media were used for the primary cultures (culture study) of the positive

blood cultures including 5% blood-sheep agar, Levin agar (Eosin-methylene blue), CHROMagar Candida, and enriching liquid medium – thioglycolate broth. The cultivation was performed in an aerobic environment. The incubation of the primary cultures was performed at 35°C ± 2°C for 24 h, after which the sample was read. With a positive culture finding, the pathogen was identified by routine biochemical tests (plasma coagulase test, catalase test, optochin test, oxidase test, indole, methyl red, Voges–Proskauer, Urea, Citrate, etc.), semi-automated (Analytical Profile Index – API, bioMerieux, France), and automated systems (VITEK-2 Compact System and VITEK MS PLUS, bioMerieux, France).

Statistical analysis

Systematization, processing, and analysis of primary data as quantitative and qualitative variables were performed with the statistical package software for the social sciences SPSS Statistics v. 22 (IBM, USA). For all tests, $p < 0.05$ was considered statistically significant. The analysis, conclusions, and recommendations of the study were outlined in a summary presentation of empirical results in tabular form and illustrated by graphic images. Graphical analysis was performed using MS Office 365 using Excel.

Results

General characteristics of the studied patients

The study included 40 men (55.6%) and 32 women (44.4%) with a mean age of 37.4 ± 3.5 years. The most commonly studied patients were aged 0−14 years and 60−74 years (Figure 1).

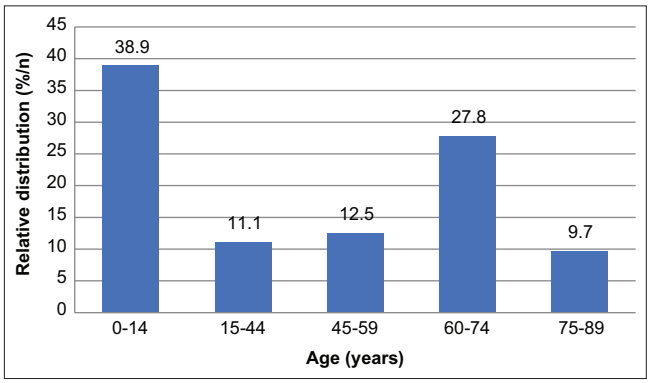

Figure 1: Relative distribution by age of the patients tested with FISH

The patients were most commonly from clinics of pediatrics, anesthesiology and intensive care unit (ICU), hematology and oncology, cardiac surgery, etc. (Figure 2).

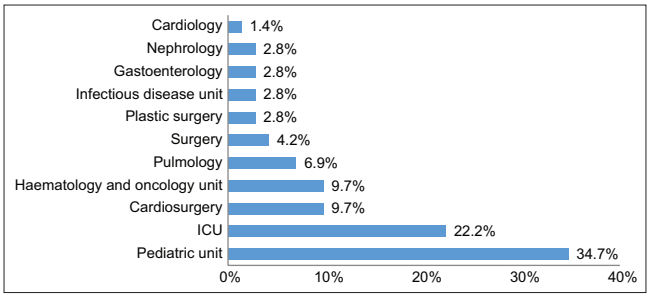

Figure 2: Relative distribution of the collected blood cultures from clinics examined with FISH

1. FISH capabilities for the identification of microorganisms from positive blood cultures

In 63 (87.5%) of the blood cultures, FISH identified microorganisms, and in 9 (12.5%) fluorescence was not observed. Its absence was because not all microorganisms were included in the test's spectrum. Gram-positive microorganisms that can be detected by the test – *Staphylococcus aureus,* CoNS, *Enterococcus faecium,* and *Enterococcus faecalis,* had a leading role as causative agents of bacteremia – n = 38 (52.8%), followed by Gram-negative microorganisms (n = 18, 25.0%), and fungi (n = 6, 8.3%). In one case, mixed infection of Gram-negative microorganisms was found – *K. pneumoniae* and *P. aeruginosa* (Figure 3).

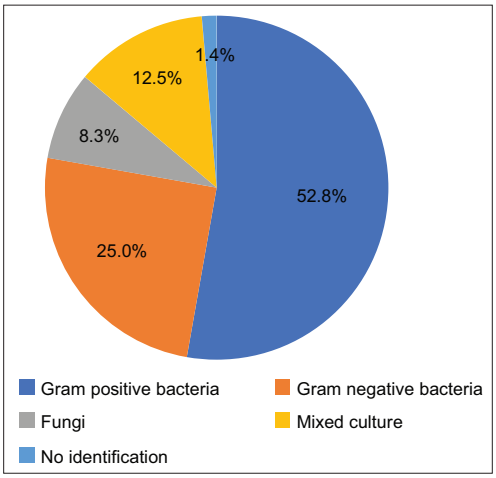

Figure 3: Group distribution of detected microorganisms by FISH test

S. aureus was detected in 10 (15.9%) of the studied blood cultures, and coagulase-negative staphylococci (CoNS) – in 20 (31.6%). Using FISH, we were able to quickly distinguish *S. aureus* from CoNS. It was found that this method made it possible to distinguish *Staphylococcus* species that were plasma agglutination-negative, but plasma coagulase-positive from *Staphylococcus* species that were negative for both tests – rabbit plasma agglutination and coagulation. *E. faecalis* was detected in 4 (6.4%) of the tested samples and *E. faecium* in 4 (6.4%).

Among the Gram-negative microorganisms as causative agents of bacteremia, the most common was

E. coli (n = 7; 11.1%), followed by *P. aeruginosa* (n = 6; 9.5%) and *K. pneumoniae* (n = 5; 7.9%).

Yeast infections caused by *C. albicans, C. glabrata,* or *C. krusei* can be successfully identified by the method. Fungemia was found in six cases. *C. albicans* (n = 4; 6.4%) and *C. parapsilosis* (n = 2; 3.2%) were detected by confirmatory methods. Figure 4 shows the distribution of the detected microorganisms by the FISH test.

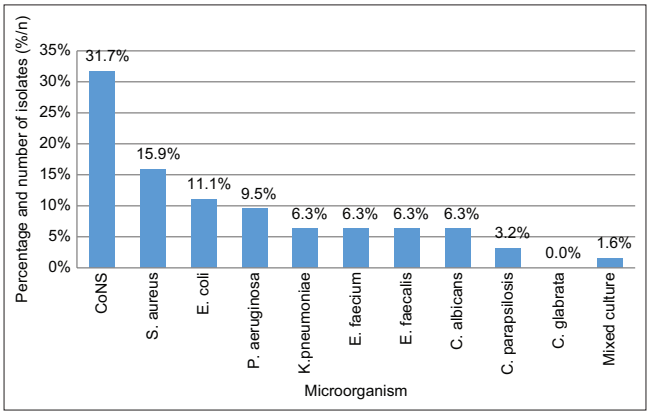

Figure 4: Distribution of species isolates from blood cultures identified with QuickFISH BC

2. A comparative study with routine microbiological methods of positive blood cultures

All 63 blood cultures from which microorganisms were proven by QuickFISH ™ were confirmed by conventional test methods. 100% comparability of the results obtained through the routine identification and QuickFISH BC method was found. The obtained images and colors of fluorescence were easy to interpret and identify the microorganisms (Figure 5).

In the nine cases with lack of identification through QuickFISH™, we identified using classical microbiological diagnostic methods microorganisms such as *Streptococcus spp., Acinetobacter baumannii, Kocuria kristinae, Pantoea spp., Klebsiella aerogenes (formerly Enterobacter aerogenes)*, *Streptococcus mutans, Enterobacter cloacae* complex, *Shewanella putrefaciens,* and *Candida lusitaniae*, which were not in the spectrum of the QuickFISH BC test. The test sensitivity to all studied cultured microorganisms was 84.5%. The performed statistical analysis demonstrates that FISH is a reliable method of rapid identification of microorganisms due to the large relative share of coincidence of the identified microorganisms with this method compared to the routine tests (z = 4.80, p = 0.001).

Discussion

Microbiological laboratories require rapid, reliable, and cost-effective methods for identifying potential pathogens in clinical samples so that adequate

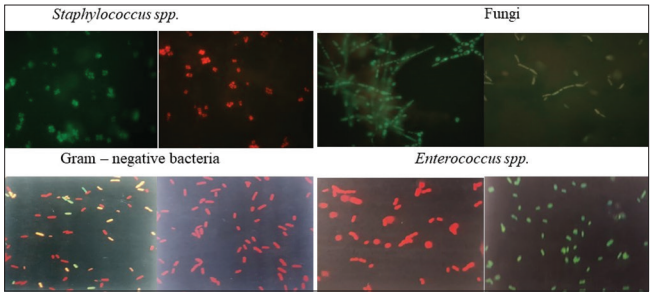

Figure 5: Photos of received images from QuickFISH BC

antimicrobial therapy can be initiated over time [20]. Quick identification of pathogens in the bloodstream is essential for improving survival in patients with BSIs. Fluorescent *in situ* hybridization is a modern technique for the rapid identification of pathogens from the blood. The test has not yet been validated for specimens other than blood, but depending on high sensitivity, its applications for pleural, peritoneal, and ascites samples have being discussed.

QuickFISH BC accelerates the identification of the most common bacteria and fungi (10 species), responsible for bacteremia, and fungemia. The results obtained with this method showed a 100% match to conventional methods for blood culture analysis, which is also confirmed by other authors [10], [11], [12], [13], [14]. The ability to differentiate coagulase-negative staphylococci (CoNS) quickly and accurately is important, because they are responsible for 20% of all cases of bacteremia, especially in immunocompromised patients. In most cases, they are skin contaminants. Despite this fact, their identification can lead to the initiation of unnecessary therapy for the patient. On the other hand, according to *Stender et al.*, the timely identification of *S. aureus* by the FISH method can improve the empirical treatment and thus lead to an overall reduction in the use of antibiotics, including vancomycin, usually empirically given for "therapy" in patients with bloodstream infections [15]. This, in turn, can help reduce the spread of nosocomial infections caused by antibiotic-resistant bacteria, especially vancomycin-resistant enterococci as it is claimed also in the studies of Buehler *et al.,* Oliveira *et al.,* and Koncelik *et al*. [12], [16], [17].

FISH can complement traditional microbiological methods for faster and timely identification of the leading microorganisms causing bacteremia/fungemia with the help of fluorescent PNA probes targeting 16S rRNA of bacteria and 18S rRNA of fungi [15], [16], [18]. We encountered difficulties in the primary microscopic differentiation between *Staphylococcus* spp. and *Enterococcus spp.* For this reason, we applied the QuickFISH BC tests for enterococci and staphylococci simultaneously, where green or red fluorescence was observed at the respective specific target slide.

Kempf *et al*. assert that the probes used in FISH are highly specific and hybridized only to the target strain, which we support with the present study.

Thus, with FISH, depending on the group of the studied microorganisms (bacteria or fungi), a time saving of 26−46 h is achieved, compared to the traditional laboratory methods used for identification. This is also confirmed in our study. Despite that, to consider, the cost-effectiveness of the test should do other comparative studies with a large number of strains. According to Kempf *et al*. and Koncelik *et al*., this leads to a correction of the antimicrobial treatment of these patients 1−2 days earlier [17], [19]. Seo *et al.* claim that the introduction of QuickFISH leads to greater avoidance of patients' therapy with vancomycin [20].

At the same time, without identification of the pathogen by this method, it is necessary to continue the investigation with other methods, which given the limited spectrum of the included microorganisms. We also encountered these difficulties in identification in our study. This indicates that QuickFISH BC can be used in addition to standard microbiological testing. Several studies have reported that FISH is a rapid and reliable method for direct identification and differentiation of bacteria and yeast from positive blood cultures, inexpensive (about $ 20 for the sample), validated, and suitable for daily routine work [11], [16], [19]. These data correlate with the results of our study and prove FISH as fast (the time to run and analyze the sample is about 45 min) and accurate identification method. However, the narrow spectrum (ten microorganisms) and the relatively high cost of the sample in Bulgaria (approximately 40 BGN) may prevent its imposition as a routine method. Fluorescent *in situ* hybridization (FISH) accelerates the identification of the most common causative agents of BSI. FISH can serve as a useful tool to complement traditional microbiological methods for faster and more timely identification of microorganisms causing bacteriemia/fungemia [18].

Two recent studies have shown that the use of PNA FISH probes to identify fungi in positive blood cultures reduces the costs for patients by applying appropriate antimicrobial therapy. Rapid results allow a significant reduction in the use of caspofungin with total cost savings of $1,729 per patient. In a similar study, Alexander *et al*. used an analytical model for decision-making, which proved average savings of $1837 per patient using PNA FISH [21]. These studies demonstrate that the introduction of such rapid and accurate methods for the identification of microorganisms from positive blood cultures not only contributes to the correct therapeutic approach and the outcomes but also reduces hospitalization costs and impedes the development of antimicrobial resistance.

Conclusion

The implementation of new methods such as FISH is associated with fast and reliable results for the detection of the most common bacteria and fungi that cause BSIs, immediately after a positive blood culture. Due to its relatively narrow spectrum for the identification of microorganisms, FISH may be employed in addition to routine microbiological testing for the early detection of the causative agent and aids the adequate assessment of the therapy and patient's condition. The initiation of accurate and adequate antimicrobial therapy significantly helps to prevent lethal outcomes or complications in patients, as well as reducing hospital stay.

Ethics Approval

All procedures performed in the presented study were in accordance with the ethical standards of the institution and with the 1964 Helsinki Declaration and its later amendments. Written informed consent ware obtained from all patients.

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