J PREV MED HYG 2012; 53: 104-108

SHORT ARTICLE

Molecular approaches in the diagnosis of sepsis in neutropenic patients with haematological malignances

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Key words

Epidemiology • Infection • Real-time PCR • Blood culture • Neutropenia • Sepsis

Summary

Introduction. *Sepsis is a major cause of significant morbidity and mortality in neutropenic patients. Blood culture remains the gold standard in the microbiological diagnosis of bacterial or fungal bloodstream infections, but it has clear limits of rapidity and sensitivity. The objective of the study was to compare the real-time polymerase chain reaction (RT-PCR) with automated blood cultures (BC) method in detection in whole blood of pathogens in febrile neutropenic patients with hematological malignancies.*
Methods. *A total of 166 consecutive febrile neutropenic patients were enrolled. Blood samples for cultures and SeptiFast testing were obtained at the onset of fever, before the implementation of empirical antibiotic therapy.*
Results. *Forty (24.1%) samples out of the 166 blood samples tested, were positive by at least one method. Twenty-three (13.9%) samples were positive by blood culture and 38 (22.9%) by multiplex real-time PCR. The analysis of concordance evidenced a low correlation between the two methods (n = 21; 52.5%), mainly due to samples found negative by culture but positive with the SeptiFast assay. Sensitivity, specificity, and positive and negative predictive values of RT-PCR were 91.3%, 88.1%, 55.3%, and 98.4%, respectively, compared with BC.*
Discussion. *Multiplex real-time PCR assay improved detection of the most bacteria associated with febrile neutropenia episodes. Further studies are needed to assess the real advantages and clinical benefits that molecular biology tests can add in diagnosis of sepsis.*

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Introduction

The sepsis is a leading, infectious complication among critically ill patients and represents a substantial health care burden [1]. It is the combination of pathologic infection and physiological changes collectively known as the systemic inflammatory response syndrome [2]. The incidence of sepsis and the number of sepsis-related deaths are increasing, and is now among the 10 leading causes of death in the Unites States with > 700,000 cases per year and with a mortality rate of around 29% [3]. Recent data have estimated that the incidence rate for severe sepsis lies between 50 and 100 cases per 100,000 individuals among industrialized countries [4].
The diagnosis is made on the basis of the presence of inflammatory response indicators in the setting of suspected or confirmed infection. The sepsis syndromes are a continuum of a disease process progressing from sepsis (infection with an inflammatory response) to severe sepsis (sepsis with organ dysfunction) to septic shock (sepsis with tissue hypoperfusion) [3].
Possible reasons of the increased incidence of sepsis include massive use of invasive procedures and immunosuppressive drugs, chemotherapy, and transplantation; the spread of human immunodeficiency virus (HIV) infection and increasing microbial resistance [5].
Coagulase-negative Staphylococci (CoNS), *Staphylococcus aureus (S. aureus), Enterococcus sp.*, Gram-negative bacilli and *Candida sp.* represent the most commonly isolated pathogens in these infections [6].
However, the microbiology of sepsis has changed over time. Although in the past gram negative organisms were most commonly implicated, increasingly gram-positive organisms are isolated [7], such that roughly similar numbers of gram-positive and gram-negative organisms are now associated with sepsis. Sepsis can also be caused by a fungal or parasitic infection, and no infectious agent is identified in about one-third of patients [8].
Patients with a history of cancer are at increased risk for acquiring and subsequently dying from sepsis, compared to the general population, although incidence and fatality rates are decreasing over time [9].
It is clinically difficult to recognize sepsis because signs and symptoms are often non-specific. Fever is a known cardinal sign of sepsis and has been for many years considered a harmful effect and widely treated with antipyretic agents. The possible infectious origin of fever is a key point in the management of neutropenic patients. Indeed, febrile neutropenia is associated with reduced survival, due not only to the infectious complications, but also to other factors, including chemotherapy dose-delays and reductions [10]. Prompt diagnosis of infec-

tion in febrile neutropenia patients with hematological malignancy is essential for choosing the appropriate therapeutic strategy.
Blood culture remains the gold standard for diagnosing microbial pathogens in the blood, but that has clear limits of rapidity and sensitivity [11].
Aim of the study was to compare the real-time polymerase chain reaction (RT-PCR) with automated blood cultures (BC) method in detection in whole blood of pathogens most frequently responsible for infections of the bloodstream in febrile neutropenic patients with hematological malignancies.

Methods

Patients and specimens. A total of 166 consecutive febrile neutropenic patients (median age 66.1 years, range 23-82 years; 103 males and 63 females) were enrolled between January 2010 and December 2010. All patients were admitted to the Hematology Department, Hospital "G. Panico", Tricase, Italy. The blood samples were drawn from febrile (temperature > 38.0°C) neutropenic patients with a neutrophil count below $< 0{,}5 \times 10^9\ L^{-1}$ in presence of acute and/or chronic blood disorders or bone marrow transplant. Blood samples for cultures and Septi*Fast* testing were obtained at the onset of fever, before the implementation of empirical antimicrobial therapy. Febrile episodes in these patients were therapeutically managed at the physician's discretion on the basis of the suspected etiology, local microorganism resistance patterns and the severity of illness.
Cultural methods. Blood cultures were done using the Automated BacT/Alert 3D (bioMérieux, France) continuous-monitoring blood culture system, following the instructions of the manufacturer. 10 ml of peripheral venous blood were collected from patients, and immediately inoculated into two bottles BacT/Alert (bioMérieux, France), one for aerobic microorganisms and one for anaerobes. The bottles were then processed in a BacT/Alert 3D automated blood culture system, with monitoring of carbon dioxide production within each bottle every 10 min 24 h per day. From 8 am to 7 pm, all bottles signalled as positive were removed from the instrument, and an aliquot was taken for Gram stain and culture on solid media for subsequent analysis. Identification and determination of sensitivity to antibiotics were performed with the VITEK 2 system (bioMérieux, France).
Molecular methods. For each sample, 1.5 ml K-EDTA-treated uncultured blood was processed by the commercial real-time PCR test LightCycler Septi*Fast* (Roche Molecular System Branchburg, NJ, USA). LightCycler SeptiFast Test is an assay capable of detecting a wide range of bacterial and mycotic pathogens (Tab. I).
The LightCycler Septi*Fast* MGRADE test was performed on blood samples, as described in several recent publications [12, 13].

Results

Forty (24.1%) samples out of the 166 blood samples tested were positive by at least one method. Twenty-three (13.9%) samples were positive by blood culture and 38 (22.9%) by multiplex real-time PCR (Tab. II). The analysis of concordance evidenced a low correlation between the two methods (n = 21; 52.5%), mainly due to samples found negative by culture but positive with the Septi*Fast* assay. Conversely, only 2 out of 23 samples positive by blood culture (8.7%) were negative by real-time PCR (Tab. II).
Sensitivity, specificity, and positive and negative predictive values of RT-PCR were 91.3%, 88.1%, 55.3%, and 98.4%, respectively, compared with BC.
Gram-negative bacteria were the most frequently isolated pathogens (n = 27; 67.5%), followed by Gram-positive (n = 9; 22.5%) and fungi (n = 4; 10.0%) (Tab. III).
Only for some species (CoNS, *Escherichia coli, Serratia marcescens* and *Candida parapsilosis*) high concordance of methods was found; while for all other species only Septi*Fast* was able to detect the presence of pathogens in blood samples (Tab. III).

Tab. I. List of pathogen detectable by LightCycler Septi*Fast* Test.

Gram-positive bacteria	Gram-negative bacteria	Fungi
Staphylococcus aureus	*Escherichia coli*	*Candida albicans*
Coagulase-negative staphylococci	*Klebsiella pneumoniae/oxytoca*	*Candida tropicalis*
Staphylococcus hominis	*Serratia marcescens*	*Candida krusei*
Staphylococcus epidermidis	*Enterobacter cloacae/aerogenes*	*Candida glabrata*
Streptococcus pneumoniae	*Proteus mirabilis*	*Candida parapsilosis*
Streptococcus spp	*Pseudomonas aeruginosa*	*Aspergillus fumigatus*
Streptococcus pyogenes	*Acinetobacter baumannii*	
Streptococcus agalactiae	*Stenotrophomonas maltophilia*	
Streptococcus mitis		
Enterococcus faecium		
Enterococcus faecalis		

Tab. II. Comparison of results from the LightCycler SeptiFast Test and BacT/Alert blood culture system in the cohort patients (n = 45/166) studied.

LightCycler SeptiFast Test	BacT/Alert blood culture system		Total no. of sample (%)
	Positive samples No. (%)	Negative samples no. (%)	
Positive samples (%)	21 (12.7)	17 (10.2)	**38 (22.9)**
Negative samples (%)	2 (1.2)	126 (75.9)	**128 (77.1)**
Total (%)	**23 (13.9)**	**143 (86.1)**	**166 (100)**

Tab. III. Microorganisms positive (n = 40) detected by blood cultures and SeptiFast assay.

	Blood cultures n. (%)	LightCycler SeptiFast Test n. (%)	Both assays n.
Gram-positive			
Staphylococcus aureus	0 (0.0)	3 (7.9)	3
Coagulase-negative staphylococci	0 (0.0)	1 (2.6)	1
Staphylococcus hominis	3 (13.0)	3 (7.9)	3
Staphylococcus epidermidis	1 (4.3)	1 (2.6)	1
Staphylococcus (other types)	1 (4.3)	0 (0.0)	1
Gram-negative			
Enterobacter cloacae	1 (4.3)	2 (5.3)	2
Escherichia coli	5 (21.7)	5 (13.2)	5
Klebsiella pneumoniae	0 (0.0)	3 (7.9)	3
Acinetobacter baumannii	0 (0.0)	1 (2.6)	1
Proteus mirabilis	0 (0.0)	1 (2.6)	1
Serratia marcescens	5 (21.7)	5 (13.2)	5
Stenotrophomonas maltophilia	0 (0.0)	1 (2.6)	1
Pseudomonas aeruginosa	5 (21.7)	8 (21.1)	8
Burkholderia cepacia	1 (4.3)	0 (0.0)	1
Fungi			
Aspergillus fumigatus	0 (0.0)	1 (2.6)	1
Candida albicans	0 (0.0)	1 (2.6)	1
Candida krusei	0 (0.0)	1 (2.6)	1
Candida parapsilosis	1 (4.3)	1 (2.6)	1
Total	**23 (57.5)**	**38 (95.0)**	**40 (100)**

Only in one case, repeated measurements by cultural method were negative for *Aspergillus fumigatus* but positive by molecular method.

Discussion

All patients with hematologic malignancies undergoing intensive cytotoxic chemotherapy causing long-term granulocytopenia are at high risk of infectious complications [9].

In recent decades, the management of severe neutropenic patients has significantly improved in both the prognosis and the antimicrobial treatment [14], while the etiological diagnosis is still mainly based on blood culture.

Due to the low sensibility and specificity of conventional method to diagnose the sepsis, the need for new more rapid and effective techniques became more urgent.

In recent years, the incidence of sepsis is increasing and the diagnosis in neutropenic patients and, more generally immunocompromised, remains a challenge as approximately half of sepsis cases are culture negative [15].

The increased incidence of the invasive fungal infections and of the infections caused by CoNS, as a result of the widespread use of vascular catheters, and the antimicrobial prophylaxis, are all factors that limit the ability of blood cultures to identify the causative agent of bloodstream infections in onco-hematological patients.

The delay or failure in diagnostic procedures lead to inadequate treatment in 25% of cases, with a significantly increased risk of death [16]. The autopsy confirmed that a misdiagnosis along with inadequate antimicrobial treatment is the most preventable cause of death due to sepsis.

Moreover, an inappropriate antibiotic therapy seem to be an important determinant of hospital mortality, as it may cause side effects and antibiotic resistance as well as increased costs of treating patients [17-19].

The results of blood cultures, generally, don't allow to start a correct antibiotic therapy because they are not available as soon as necessary.
Indeed, blood culture typically becomes positive 8-36 h after sampling, and therapy can then be adapted and based on presumptive bacterial identification suggested by Gram-stain characteristics. A more precise pathogen identification and susceptibility profile, however, is not available until up to 24-48 h [20, 21].
An important retrospective study showed that during severe sepsis effective antimicrobial administration within the first hour is associated with increased survival [22].
One of the most promising approaches for diagnosis of blood infections seems to be directly based on the identification of bacterial and fungal DNA in whole blood by PCR assays [12].
Overall, according to our data multiplex real-time PCR assay improved detection of the most bacteria associated with febrile neutropenia episodes. Results are similar to the previous reports comparing the lightCycler Septi*Fast* assay with the conventional blood cultures [9, 12, 13, 24].
However, although most episodes of febrile neutropenia are assumed to be caused by an infection, the incidence of proven bloodstream infections in febrile neutropenia is only ~30% based on results of blood cultures [25, 26].
Recent studies reporting a higher yield of positive results by various PCR-based methods to detect bacterial and fungal DNA have yet to be validated in larger cohorts [12, 27].
There is a limitation in order to PCR test used for identification of bacteria and fungi from whole blood specimens; in fact it is impossible to collect simultaneously data on the isolated pathogen and its antibiotic susceptibility.
The antibiotic resistant marker genes that can be identified by molecular biological methods are rapidly increasing; many of these can be identified routinely, such as mecA gene for methicillin resistance in *S. aureus* and rpoB/katG/inga for resistance to rifampicin/isoniazid in *M. tuberculosis*.
However, the detection of all the genes of resistance, evaluated in more than a hundred, would require a large number of tests for each sample.
Despite the advantages of the Septi*Fast* method, the blood culture remains an important diagnostic tool that provides essential information regarding the antibiotic resistance of microorganisms. Therefore, blood culture and real-time PCR methods are not interchangeable but should be applied in combination to get a correct diagnosis.
In conclusion, it is obvious that the blood culture remains the reference method for diagnosis of sepsis, allowing the detection of bacteria and fungi responsible of infection and the evaluation of antibiotics susceptibility. However, the simultaneous application of molecular techniques such as the Septi*Fast* with blood cultures could provide a valuable contribution to the clinician because of the rapidity of testing (6 hours), to detect co-infections or slow growing organisms, and to the ability to isolate pathogens even in patients receiving prophylactic/antibiotic therapy.
However, further studies are needed to assess the real advantages and clinical benefits that molecular biology tests can add in diagnosis of sepsis, especially in neutropenic patients with onco-hematological cancers.

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■ Received on February 27, 2012. Accepted on March 30, 2012.

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