Molecular diagnosis of sepsis in neutropenic patients with haematological malignancies

Nicasio Mancini,¹ Daniela Clerici,² Roberta Diotti,¹ Mario Perotti,¹ Nadia Ghidoli,¹ Donata De Marco,¹ Beatrice Pizzorno,³ Thomas Emrich,⁴ Roberto Burioni,¹ Fabio Ciceri² and Massimo Clementi¹

Correspondence

Nicasio Mancini
mancini.nicasio@hsr.it

¹Laboratorio di Microbiologia e Virologia, Università 'Vita-Salute' San Raffaele, Milano, Italy

²Unità di Ematologia e Trapianto di Midollo Osseo, Istituto Scientifico San Raffaele, Milano, Italy

³Roche Diagnostics S.p.A., Monza, Italy

⁴Roche Diagnostics GmbH, Molecular Diagnostics, Penzberg, Germany

The rapid diagnosis of an infectious cause in the course of fever of unknown origin plays a pivotal role in the correct management of neutropenic patients. In this study, blood samples from febrile oncohaematological patients were tested using a novel commercial real-time PCR assay (LightCycler SeptiFast; Roche Molecular Systems) and blood culture (BacT/Alert 3D; bioMérieux). Twenty-one (20.4 %) and 34 (33 %) of the 103 samples under study tested positive by blood culture and PCR, respectively. The analysis of concordance evidenced a low correlation between the two approaches (83 %), mainly due to samples that tested negative by culture but positive using the molecular approach. Among 14 discordant cases negative by culture but positive by PCR, 12 were observed in sequential samples of patients with initial concordant results on samples drawn before the administration of a specific antimicrobial therapy. Moreover, DNA of a fastidious organism, *Aspergillus fumigatus*, not easily detectable by the cultural approach was rapidly detected in the two remaining discordant cases. Overall, the characteristics featured by the molecular method could be of interest in the development of new algorithms for the diagnosis of sepsis in critical patients.

Received 29 October 2007

Accepted 28 January 2008

INTRODUCTION

The possible infectious origin of fever is a central 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 (Lo & Cullen, 2006). Indeed, infections are a major risk for neutropenic patients, with the incidence of infections being related to the degree and duration of neutropenia (Norgaard *et al.*, 2006). A rapid microbiological diagnosis could therefore confirm an infectious cause of fever (thereby excluding non-infectious causes) and help in the choice of a specific therapy. Among the infectious causes, bacteria and fungi are the leading threats, with high infection-related mortality rate, especially for polymicrobial infections and moulds (Norgaard *et al.*, 2006; Wisplinghoff *et al.*, 2003). The current gold standard for the detection of bacterial pathogens in blood is blood culture. However, all blood culture systems suffer from several limitations, such as lack of rapidity and low sensitivity, especially when the patient has already received antibiotics and when fastidious micro-organisms are involved (Peters *et al.*, 2004). From this perspective, the diagnosis of bloodstream infections could

prove really challenging in oncohaematological patients, who routinely receive prophylactic antibiotics and whose blood cultures therefore often remain negative (Buchheidt *et al.*, 2003; Norgaard *et al.*, 2006; Serody *et al.*, 2000; Wisplinghoff *et al.*, 2003). Even after the detection of growth in cultured blood (usually not before 6–12 h of incubation), conventional blood cultures require at least a further 24–48 h for the definitive identification of the pathogen and the evaluation of its sensitivity to antibiotics (Beekmann *et al.*, 2003; Peters *et al.*, 2004). Other parallel approaches are therefore needed, and among them well-designed molecular assays could prove really useful. Several molecular techniques have already been successfully used in routine microbiology laboratories for direct detection of viral, bacterial, mycotic and protozoan pathogens (Nolte & Caliendo, 2007). However, their use on whole blood samples for detection of sepsis has been hampered by several factors, including insufficient sensitivity, presence of PCR inhibitors in blood, and the difficulty of setting up an assay capable of detecting a wide range of potential pathogens (Peters *et al.*, 2004).

In the present study, we evaluated a novel molecular strategy for the diagnosis of sepsis directly from blood

samples (LightCycler SeptiFast Test M Grade; Roche Molecular Systems), comparing its features to those of an automated continuous-monitoring conventional blood culture system (BacT/Alert 3D; bioMérieux). LightCycler SeptiFast Test is a real-time PCR-based assay reportedly capable of detecting a wide range of bacterial and mycotic pathogens (Table 1). The assay uses dual fluorescence resonance energy transfer probes targeting the species-specific internal transcribed spacer (ITS) regions. These regions are multicopy non-coding sequences interspaced among highly conserved bacterial and fungal rDNA that have already been used as target for the identification of microbial pathogens (De Marco *et al.*, 2007; Lau *et al.*, 2007; Mancini *et al.*, 2005, 2006; Park *et al.*, 2006; Pryce *et al.*, 2003).

METHODS

Patients and specimens. A total of 103 blood samples were collected from 34 consecutive patients admitted to the Haematology Unit of San Raffaele Scientific Institute, Milan, Italy. Over a period of about 2 months (15 January–21 March 2007), each time a blood culture was requested (collecting at least 20 ml blood), a small aliquot of blood (1.5 ml) was also sampled for the molecular assay. Seventy-three (70.9%) samples were drawn from heavily neutropenic patients with a neutrophil count below $0.5 \times 10^9 \text{ l}^{-1}$. Ninety-five (92.2%) samples were collected from patients with fever of unknown origin ($T > 38^\circ \text{C}$), whereas eight (7.8%) were surveillance samples drawn from central venous catheters. Other clinical characteristics of enrolled patients are presented in Table 2.

Cultural approach. For each sample, an average of 25 ml blood was inoculated into BacT/Alert FAN aerobic and anaerobic bottles. 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), and required not less than 24–36 h.

Molecular approach. For each sample, 1.5 ml K-EDTA-treated uncultured blood was processed for the SeptiFast assay. Using this method, the blood sample was mechanically (glass and ceramic beads) and chemically (guanidinium thiocyanate and proteinase K) lysed; the purified DNA was then bound on glass fibre and eluted at high temperature. During the study, in order to minimize the risk of contamination, all extraction procedures were performed in a dead air box situated in a dedicated pre-PCR room. The extracted DNA was then amplified, and the specific melting profile of amplified products was calculated by dedicated software (SeptiFast Software Set), thus allowing the detection of the pathogen and its identification at the genus, and often also at the species, level. During the study, the availability of a single 30-capillary LightCycler 2.0 instrument allowed the simultaneous processing of up to eight samples for the detection of Gram-positive and Gram-negative bacteria and fungi, including a positive and a negative control for each panel. The reported turnaround time of the assay is approximately 6 h from the beginning of the extraction phase (with an operator time requirement of about 3 h), thus allowing two distinct daily runs of the assay with results at 4 pm (starting the extraction phase at 10 am) and 9 am the following day (starting the extraction phase at 4 pm, and an overnight amplification). Once collected, all samples were immediately sent to the laboratory by a pressurized air system that allows the rapid delivery of samples and kept at room temperature until processed. Most samples were collected between 8 am and 9 am and therefore were processed after a maximum of 2 h. The results obtained with the molecular assay were not communicated to the laboratory staff performing the cultural approach.

RESULTS AND DISCUSSION

Among the 103 blood samples, 35 (34%) samples were positive by at least one of the two methods. Twenty-one (20.4%) samples were positive by blood culture and 34 (33%) with SeptiFast (Table 3). The analysis of concordance evidenced a low correlation between the two approaches

Table 1. Panel of pathogens detectable by the LightCycler SeptiFast Test M Grade

Gram-negative bacteria	Gram-positive bacteria	Fungi
<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>	<i>Candida albicans</i>
<i>Klebsiella (pneumoniae/oxytoca)</i>	Coagulase-negative staphylococci*	<i>Candida tropicalis</i>
<i>Serratia marcescens</i>	<i>Streptococcus pneumoniae</i>	<i>Candida parapsilosis</i>
<i>Enterobacter (cloacae/aerogenes)</i>	<i>Streptococcus</i> spp.†	<i>Candida glabrata</i>
<i>Proteus mirabilis</i>	<i>Enterococcus faecium</i>	<i>Candida krusei</i>
<i>Acinetobacter baumannii</i>	<i>Enterococcus faecalis</i>	<i>Aspergillus fumigatus</i>
<i>Pseudomonas aeruginosa</i>		
<i>Stenotrophomonas maltophilia</i>		

*The following staphylococcal pathogens are detected as a group by a single dual fluorescence resonance energy transfer probe: *S. epidermidis*; *S. haemolyticus*; *S. hominis* (subsp. *hominis/novobiosepticus*); *S. pasteurii*; *S. warneri*; *S. cohnii* (subsp. *urealyticum/cohnii*); *S. lugdunensis*; *S. capitis* (subsp. *urealyticus/capitis*); *S. saprophyticus*; *S. saprophyticus* subsp. *saprophyticus*; *S. xylosus*.

†The following streptococcal pathogens are detected as a group by a single dual fluorescence resonance energy transfer probe: *S. pyogenes*; *S. agalactiae*; *S. mitis*; *S. anginosus*; *S. bovis*; *S. constellatus*; *S. cristatus*; *S. gordonii*; *S. intermedius*; *S. milleri*; *S. mutans*; *S. oralis*; *S. parasanguinis*; *S. salivarius*; *S. sanguinis*; *S. thermophilus*; *S. vestibularis*.

Table 2. Clinical characteristics of the 34 patients involved in the study

AML, Acute myeloid leukaemia; ALL, acute lymphoblastic leukaemia; HD, Hodgkin's disease; NHD, non-Hodgkin's disease; MDS, myelodysplastic syndrome; CLL, chronic lymphocytic leukaemia; MM, multiple myeloma; Biph. AL, biphenotypic acute leukaemia; ANC, absolute neutrophil count.

No. patients	34
No. samples	103
Mean age, years (range)	47 (21–69)
Sex, no. (%)	
M	23 (67.6)
F	11 (32.4)
Underlying disease, no. (%)	
AML	14 (41.2)
ALL	5 (14.7)
HD	5 (14.7)
NHD	3 (8.8)
MDS	2 (5.9)
CLL	2 (5.9)
MM	2 (5.9)
Biph. AL	1 (2.9)
Treatment, no. (%)	
Chemotherapy with or without autologous stem cell infusion	15 (44.1)
Allogeneic stem cell transplantation	12 (35.3)
Other treatments	7 (20.6)
Neutropenia WHO grades, no. of samples (%)	
IV (ANC $<0.5 \times 10^9 \text{ l}^{-1}$)	73 (70.9)
III (ANC $<0.75 \times 10^9 \text{ l}^{-1}$)	5 (4.9)
III (ANC $<1 \times 10^9 \text{ l}^{-1}$)	25 (24.2)

(83 %), mainly due to samples that tested negative by culture but positive using the molecular approach. Indeed, 12 out of the 14 cases negative by culture but positive by PCR were observed in sequential samples of patients with initial concordant results on samples drawn before the administration of a specific antimicrobial therapy, thus excluding

Table 3. Comparison of results from the LightCycler SeptiFast Test and BacT/Alert 3D blood culture system

Reported data do not take into consideration concordance of detected micro-organisms.

LightCycler SeptiFast Test M Grade	BacT/Alert 3D blood culture system		Total no. of samples (%)
	Positive samples (%)	Negative samples (%)	
Positive samples (%)	20 (19.4)	14 (13.6)	34 (33)
Negative samples (%)	1 (0.97)	68 (66)	69 (67)
Total (%)	21 (20.37)	82 (79.6)	103 (100)

the possibility of false-positive samples due to contamination. Only in a single patient already under treatment with vancomycin was a single sample positive by PCR for *Enterococcus faecium* not paralleled by any isolation in culture. Moreover, as described later, in the other discordant case the genome of *Aspergillus fumigatus* was detected.

Among positive samples, the concordance was very high in terms of identification at the species level, as shown in Table 4. Importantly, the molecular approach allowed the detection of *A. fumigatus* in two patients (one with a mixed bacterial and fungal infection) with clinically suspected invasive fungal infections. This diagnosis was further confirmed in both patients, after almost a week, by growth of the mould from bronchoalveolar lavages. In both patients, blood cultures were persistently negative, and levels of galactomannan antigen were continuously borderline.

Finally, the turnaround time for detection of blood culture isolates and definitive identification of the pathogen was compared to the 6 h needed for the LightCycler SeptiFast Test. As reported in Table 4, even in the two observed cases of fast-growing *Escherichia coli*, a mean of 10 h of incubation was needed, with definitive identification after an additional 36 h. The time advantage was also more important for negative samples (6 h vs 5 days), given also the very high concordance of samples negative both by PCR and by culture (68/69; 98.6 %). The single discordant case was observed in one bottle positive for a probable contaminant *Staphylococcus epidermidis* out of three sampled from the same patient.

To the best of our knowledge, this is the first study comparing a molecular approach for the diagnosis of sepsis, with high potential in terms of wide range of detectable pathogens, sensitivity and routine practicability, to a well-known routine classical cultural approach. In the cohort studied, the molecular assay demonstrated several potential advantages that could make it a useful complement to blood culture for the diagnosis of sepsis. Indeed, the molecular method (i) proved to be at least as sensitive as culture, (ii) allowed a faster identification of pathogens, (iii) allowed an even faster identification of negative samples, (iv) was not influenced by ongoing therapy and (v) allowed the rapid detection and identification from blood of fastidious organisms such as *A. fumigatus*. Overall, the study reported here has addressed a limited group of critical febrile neutropenic patients; studies including larger numbers of patients and patients with different clinical conditions are clearly necessary to validate conclusively this diagnostic methodology. Moreover, although a specific analysis of the assay's cost was not performed in this study (in terms of cost of reagents and of laboratory work), it is possible to speculate that a faster diagnosis could result, especially in critical patients, in a more rational use of antibiotics and antimycotics and therefore also in a possible economical advantage. Indeed, a careful costs/benefits analysis is

Table 4. Comparative yields of bacterial and fungal species isolated during the study

Mean time needed for detection and definitive identification at the genus or species level for blood culture isolates is also reported.

Micro-organism	No. of isolates			Mean time (h) (95% CI) for:	
	Positive with both systems	Positive only with SeptiFast	Positive only with blood culture	Detection with blood culture	Definitive identification
CoNS*	12	3	1	22.2 (14.5–29.9)	44.2 (36.5–51.9)
<i>E. faecalis</i>	5	4	0	12.6 (8.5–16.6)	56.6 (52.5–60.7)
<i>E. faecium</i>	0	1	0	NA	NA
<i>E. coli</i>	2	0	0	10	54
<i>P. aeruginosa</i>	1	5	0	15	55
<i>A. fumigatus</i> †	0	2	0	NA	NA
Total	20	15	1		

*Coagulase-negative staphylococci.

†Diagnosis of *A. fumigatus* invasive infection was confirmed by clinical data and isolation from bronchoalveolar lavage.

certainly necessary to plan new diagnostic strategies for febrile neutropenic patients. In conclusion, even if a sensitive molecular method cannot completely substitute for blood culture in the near future, the data obtained in this study strongly suggest that molecular assays may help in the development of new algorithms for the diagnosis of sepsis in critical patients.

ACKNOWLEDGEMENTS

This study was partially supported by an institutional grant from Roche, Diagnostic Division.

REFERENCES

- Beekmann, S. E., Diekema, D. J., Chapin, K. C. & Doern, G. V. (2003). Effects of rapid detection of bloodstream infections on length of hospitalization and hospital charges. *J Clin Microbiol* **41**, 3119–3125.
- Buchheidt, D., Bohme, A., Cornely, O. A., Fatkenheuer, G., Fuhr, H. G., Heussel, G., Junghanss, C., Karthaus, M., Kellner, O. & other authors (2003). Diagnosis and treatment of documented infections in neutropenic patients – recommendations of the Infectious Diseases Working Party (AGIHO) of the German Society of Hematology and Oncology (DGHO). *Ann Hematol* **82** (Suppl. 2), S127–S132.
- De Marco, D., Perotti, M., Ossi, C. M., Burioni, R., Clementi, M. & Mancini, N. (2007). Development and validation of a molecular method for the diagnosis of medically important fungal infections. *New Microbiol* **30**, 308–312.
- Lau, A., Chen, S., Sorrell, T., Carter, D., Malik, R., Martin, P. & Halliday, C. (2007). Development and clinical application of a panfungal PCR assay to detect and identify fungal DNA in tissue specimens. *J Clin Microbiol* **45**, 380–385.
- Lo, N. & Cullen, M. (2006). Antibiotic prophylaxis in chemotherapy-induced neutropenia: time to reconsider. *Hematol Oncol* **24**, 120–125.
- Mancini, N., Ossi, C. M., Perotti, M., Clementi, M., Digiulio, D. B., Schaenman, J. M., Montoya, J. G., McClenny, N. B., Berry, G. J. & other authors (2005). Molecular mycological diagnosis and correct antimycotic treatments. *J Clin Microbiol* **43**, 3584.
- Mancini, N., Perotti, M., Ossi, C. M., Cavallero, A., Matuska, S., Paganoni, G., Burioni, R., Rama, P. & Clementi, M. (2006). Rapid molecular identification of fungal pathogens in corneal samples from suspected keratomycosis cases. *J Med Microbiol* **55**, 1505–1509.
- Nolte, F. S. & Caliendo, A. M. (2007). Molecular detection and identification of microorganisms. In *Manual of Clinical Microbiology*, 9th edn, pp. 218–244. Edited by P. R. Murray. Washington, DC: American Society for Microbiology.
- Norgaard, M., Larsson, H., Pedersen, G., Schonheyder, H. C. & Sorensen, H. T. (2006). Risk of bacteraemia and mortality in patients with haematological malignancies. *Clin Microbiol Infect* **12**, 217–223.
- Park, H., Kim, C., Park, K. H. & Chang, C. L. (2006). Development and evaluation of triplex PCR for direct detection of mycobacteria in respiratory specimens. *J Appl Microbiol* **100**, 161–167.
- Peters, R. P., van Agtmael, M. A., Danner, S. A., Savelkoul, P. H. & Vandenbroucke-Grauls, C. M. (2004). New developments in the diagnosis of bloodstream infections. *Lancet Infect Dis* **4**, 751–760.
- Pryce, T. M., Palladino, S., Kay, I. D. & Coombs, G. W. (2003). Rapid identification of fungi by sequencing the ITS1 and ITS2 regions using an automated capillary electrophoresis system. *Med Mycol* **41**, 369–381.
- Serody, J. S., Berrey, M. M., Albritton, K., O'Brien, S. M., Capel, E. P., Bigelow, S. H., Weber, D. J., Gabriel, D. A., Wiley, J. M. & other authors (2000). Utility of obtaining blood cultures in febrile neutropenic patients undergoing bone marrow transplantation. *Bone Marrow Transplant* **26**, 533–538.
- Wisplinghoff, H., Seifert, H., Wenzel, R. P. & Edmond, M. B. (2003). Current trends in the epidemiology of nosocomial bloodstream infections in patients with hematological malignancies and solid neoplasms in hospitals in the United States. *Clin Infect Dis* **36**, 1103–1110.