Application Note

Molecular Diagnosis of Bacterial and Fungal Pathogens in Clinical Material by Broad-Range PCR and Sequencing

Keywords: Clinical studies, culture-independent diagnosis, body fluids and tissues, 16S/18S rRNA gene PCR, sequence analysis, culture negative infections, CE-IVD testing, UMD-Universal, SepsiTest™

Abstract: An overview of clinical studies is presented using CE-IVD marked 16S and 18S rRNA gene PCR and sequencing tests, SepsiTest™ and UMD, for the in-vitro diagnosis of a variety of infectious diseases. From the studies employing in total 999 samples from 614 patients the main conclusion was that the molecular tests are useful as supplements to culture diagnosis for the rapid identification of non-growing pathogens and adaptation of antibiotic therapy.

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Introduction

Culture diagnosis of blood stream infection is the standard procedure in clinical microbiology. Major limitations are the long time of culturing of up to 4 days and the low positivity (30 to 40%) [1]. Since their introduction, commercial products for culture-independent molecular diagnosis of infectious agents came into routine clinical practice as a rapid method to supplement culture [1].

Broad-range PCR together with sequence analysis is principally unlimited in the identification of bacterial and fungal pathogens present in a specimen. Rice and Madico [2] state that broad-range PCR is most beneficial in the identification of fastidious, non-cultivable and antibiotic-inhibited organisms. Molzym offers CE-marked products for the molecular *in vitro* diagnosis of pathogens in a variety of clinical materials. In a series of studies, microorganisms from more than 200 genera of bacteria, among them 86 Gram-positive and 120 Gram-negative, and 65 genera of fungi have been identified so far. The complete list of organisms is available on request.

The Broad-Range Molecular Diagnostic System

Two kits, *SepsiTest*™ and *UMD-Universal*, are available for the *in-vitro* diagnosis of pathogenic organisms in clinical material. The kits include protocols for the extraction of microbial DNA from blood (*SepsiTest*™) and other primary sterile body fluids of various volumes, including ascites, pleural, synovial, cerebrospinal fluids, broncho-alveolar lavage and pus (including also blood), as well as swabs and tissues (*UMD-Universal* [*UMD*]). The tests comprise of assays for the PCR or Real-Time PCR amplification of the hypervariable V3/V4 region of the 16S and the V8/V9 region of the 18S rRNA genes of bacteria and fungi, respectively. For pathogen identification, amplicons from positive samples are sequenced and compared to a library of more than 7,000 validated entries of the bacterial 16S rRNA gene and the 18S rRNA gene of yeasts and *Aspergillus* species. Mixed sequences of bacterial species can be resolved using the Ripseq® tool (Isentio, Norway). The time to positive or negative results is typically 4 hours, including manual DNA extraction and Real-Time PCR analysis. In the case of positive results, another 3 to 4 hours is needed for sequence analysis, depending on the sequencing system. Practically, sequencing is performed over night and the species record is available at the next morning.

Clinical Applications

Table 1 presents an overview of the clinical studies and the diseases focussed on. In the following sections a selection of studies employing *SepsiTest™/UMD* is discussed.

Septicaemia. Orszag et al. [7] performed a monitoring study employing 160 blood samples from 28 patients under extracorporeal oxygena-

Table 1: Clinical studies using *SepsiTest™/ UMD*

Infection	specimen	patients	samples	Ref.
Septicaemia	EDTA blood	187	342	3
		23	23	4
		50	50	5
		20	20	6
		28	160	7
Infective endocarditis	heart valve tissue	30	30	8
		46	46	9
Bacterial meningitis	cerebrospinal fluid	40	40	10
		18	66	11
Liver infection	liver tissue	22	22	12
Joint infection	synovial fluid, joint tissue	84	104	13
Diverse	liquids, tissues	66	96	14

tion. Under the above time-to-result consideration, the authors noted a 13 to 75 h earlier identification of pathogens, including coagulase-negative staphylococci, *Serratia marcescens*, *Staphylococcus aureus* and *Candida glabrata* than blood culture (BC) in 45% of BC- and SepsiTest™-positive patients. In the same study 25% of BC-negative samples were positive in the molecular analysis with sepsis relevant microorganisms. Failure of BC to detect the pathogens was attributed to the treatment of the patients with antibiotics before blood draws. The detection of culture-negative infections highlights an important benefit of molecular analysis.

Infective endocarditis (IE). In another study Marsch et al. [9] investigated the impact of positive molecular results employing heart valve (HV) specimens from IE patients on the antibiotic therapy. Of 46 cases with HV culture-negative results, inconsistent results from pre-operative BCs or intra-operative suspicion of IE, *UMD* diagnosed relevant infections in 7 patients by *Tropheryma whipplei*, *Streptococcus* spp. (3 cases), *Haemophilus parainfluenzae*, *S. aureus* and *Gemella bergeri*, respectively. These results initiated a change of the antibiotic treatment regime. The authors point out the significant benefit of molecular diagnosis by rapidly providing the identity of uncultured aetiologies of IE and the possibility of adapting the antibiotic treatment regime.

Bacterial/fungal meningitis. Cerebrospinal fluid samples (CSF) from 40 patients with clinical symptoms of central nervous system infections were analysed for the presence of bacteria and fungi by culture, microscopy and *UMD* [10]. In a sub-group of 20 patients with white blood cell counts of >500/µl CSF and suspect of bacterial infection, 13 patients (65%) were positive by *UMD*, whereas only 7 patients were positive by culture/microscopy (35%). Overall, 5 samples were positive by *UMD* and culture/microscopy with pathogens, including *S. pneumoniae*, *Neisseria meningitidis*, *Haemophilus influenzae*, *Klebsiella pneumoniae* and *Cryptococcus neoformans*. In 8/32 samples (25%) which were culture/microscopy negative further relevant bacteria were identified by *UMD*, including species mentioned above and additionally *S. agalactiae* and *Listeria monocytogenes*. The authors argue that molecular testing may reduce diagnostic uncertainty in patients with suspected CNS infection and may optimise antibiotic therapy and prophylaxis.

Biomarker guided 16S PCR diagnosis of bacterial meningitis. Stubljar et al. [11] conducted a prospective study enrolling 18 children with external ventricular drains and clinical signs of bacterial meningitis/ventriculitis. CSF samples were collected for the analysis of sCD14-ST as inflammatory biomarker and aetiologies by *UMD*. In a group of patients with ventriculitis, pathogens were identified in 37 out of 38 CSF samples (97%) by *UMD* in comparison to 17 cases by culture methods (45%). More bacteria (20 species) and mixed infections (10 samples) were diagnosed with *UMD* than culture (10 species; 6 samples mixed infections). The authors conclude that sCD14-ST guided 16S rRNA gene PCR could be used in every day clinical practice to improve the aetiological diagnosis of meningitis/ventriculitis and to prescribe more appropriate antibiotics.

Pathogens in explanted liver. In their prospective study employing paediatric patients receiving liver transplantation, Schukfeh et al. [12] analysed the spectrum and incidence of pathogens in explanted liver tissue samples. The overall positivity by culture and/or *UMD* was 9/20 (41%). With *UMD* one or more pathogens were detected in 7 (35%) and with culture in 2 (10%) patients. *UMD* identified single infections by bacteria (*S. epidermidis*, *Pseudomonas* spp., Dermacoccaceae) and fungi (*Pichia anomala*, Ascomycota) and a mixed infection by *S. epidermidis* and the yeast, *C. albicans*. The authors reasoned that universal PCR appears to be an effective detection method for the broad spectrum of pathogens in the native livers of pediatric patients undergoing liver transplantation.

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