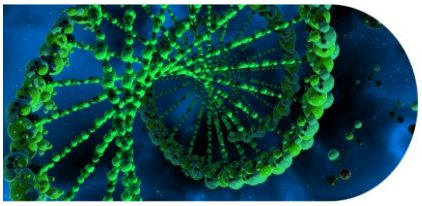

White Paper

No. 1/2021: 1-8

Clinical Applications of Molzym's Broad Spectrum Molecular Diagnostics

Culture-independent & broad-range molecular diagnostic test for the identification of bacteria and fungi overcomes limitations of culture-based methods, increases positivity rate and demonstrates clinical added value for culture-negative infections.

Introduction

Culture diagnosis is a standard method for the identification of infectious agents in clinical microbiology, although the long time to result is a commonly reported limitation [1]. In clinical practice, the administration of broad-spectrum antibiotics is often necessary for serious infections until definitive information on species identity is made available to better target therapy. Another limitation attributed to conventional culture in clinical practice is linked to its low positivity rate mostly due to initial empiric administration of antibiotics, specific growth requirements of fastidious microorganisms or uncultivable bacteria or fungi.

Modern molecular biological methods have become routinely used to detect microorganisms by analyzing their DNA - without the need for cultivation. They are considered valuable tools, complementing culture results, due to their growth independence, speed, sensitivity and reproducibility. However, they mostly still present a limitation in terms of panels targeting only a few microorganisms, which Molzym proposes to overcome with its broad-range approach, to better complement the standard methods in routine diagnostic laboratories.

In this white paper, results from multi-site studies support Molzym's Molecular Diagnostic Test (MMDx) performance as a precise and rapid tool for the identification of pathogens – growing or static - directly from samples against reference methods, in various clinical applications. The results highlight MMDx potential to optimize laboratory workflow, provide targeted and clinically relevant information to physicians, thus assisting them to improve patients' outcomes.

Molzym's Molecular Diagnostic Test

Molzym has developed culture-independent molecular test solutions (SepsiTest™-UMD, UMD-SelectNA™ and Micro-Dx™) for the *in-vitro* diagnosis of pathogens, including the most common, but also rare, fastidious and non-growing bacteria and fungi that cause sepsis, endocarditis, joint infections, peritonitis, meningitis, pyomyositis and other diseases. MMDx is based on a single protocol of microbial DNA enrichment and extraction (MolYsis™, Fig. 1), followed by broad-range PCR or Real-Time PCR and sequencing analysis. The complete 3-step diagnostic workflow of MMDx is demonstrated in Fig. 2 and the individual steps are explained in detail hereafter:

➢ *Step 1: Unique Microbial DNA Extraction*

A crucial step in direct molecular analysis is the isolation of microbial DNA from samples. The quantity and quality of bacterial and fungal DNA recovered from specimens contribute to the overall sensitivity and specificity of analytical systems [2].

In clinical samples, human DNA is in vast excess to microbial DNA, which negatively affects the analysis due to non-specific primer binding and thus severely compromises the sensitivity of the detection assay. To overcome this limitation, Molzym has developed a unique technology, MolYsis™, for the targeted isolation of microbial DNA from complex clinical samples such as body fluids, tissues or swabs, to significantly improve results and ensure highest sensitivity in subsequent analytical processes. This human DNA depletion technology takes advantage of the fact that human and microbial DNAs are present in different cell types. With MolYsis™, fragile human cells are lysed, while rigid bacterial and fungal cells are not affected due to their cell wall. Up to 99% of the released human DNA is subsequently degraded by a nuclease. Thereafter, the microbial cells are lysed and the DNA is purified. The entire workflow (Fig. 1) can be performed manually or fully automated on Molzym's SelectNA™*plus* robot.

In addition, Molzym applies certain proprietary manufacturing processes to deliver all components free of reagent- and production-related contaminations (microbes and DNA) to ensure high quality and purity of isolated microbial DNA and subsequent analysis leading to utmost sensitivity and confidence in the results.

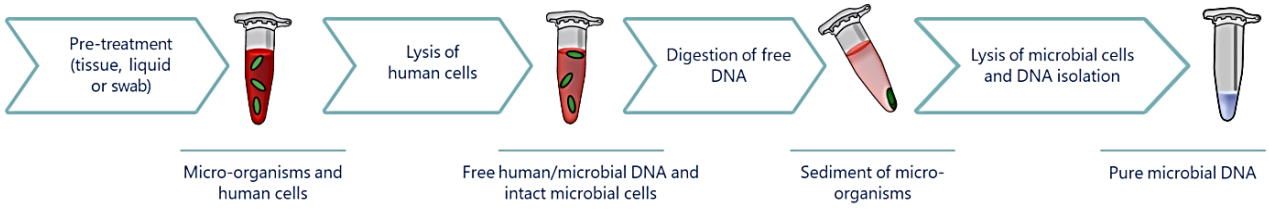

Fig. 1: MolYsis™, unique microbial DNA enrichment process

➢ *Step 2: Universal 16S and 18S PCR / Real-Time PCR*

Broad-range 16S and 18S rRNA gene PCR and sequencing are recognized methods for the accurate diagnosis of pathogens. 16S rRNA gene PCR allows comprehensive detection and identification of bacteria and 18S rRNA gene PCR enables the detection of yeasts and fungi. It is particularly useful for the etiological diagnosis of diseases that may be caused by a wide variety of organisms from very different taxa. The major strength of PCR is the detection of pathogens based on DNA independent from the growth of microbes.

The two universal PCR assays supplied with MMDx are free of contaminating microbial DNA and contain a highly active Taq DNA polymerase that ensures high amplification activity for up to 40 cycles and provides accurate results even at very low target loads. The assays can be run as PCR or Real-Time PCR setups whereby the primers bind to highly conserved sites of the genes flanking the 16S V3/V4 and 18S V8/V9 variable regions. Thus, these broad-spectrum assays have the potential to cover the entire clades of bacteria and a wide range of yeasts and fungi. Positive PCR results indicate the presence of bacterial or fungal DNA in the sample and amplicons are subsequently analyzed by Sanger sequencing.

➢ *Step 3: Sequence Analysis*

The precise identification of pathogens is carried out by Sanger sequencing analysis. For this purpose, amplicons are purified, sequenced and the sequences analyzed against a database or sequence library using a BLAST tool. From the various online available databases and platforms, some allow differentiating up to 3 bacterial species for mixed bacterial sequences.
To date, more than 1,300 bacteria and fungi at genus or species level have been identified with MMDx in clinical trials, in routine and other analyses. The complete list of identified microorganisms can be found here.

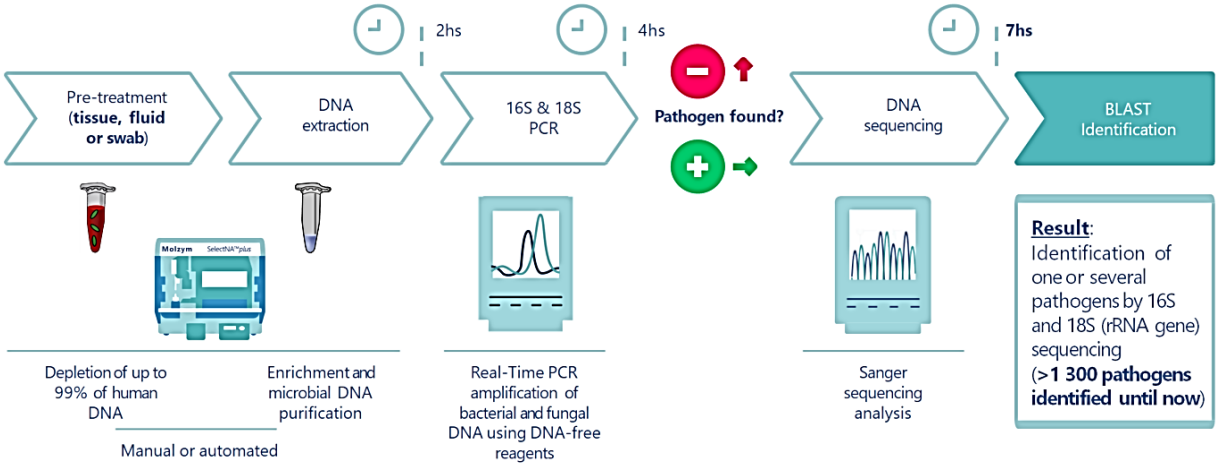

Fig. 2: MMDx broad-range diagnostic workflow

Clinical Applications of MMDx

The clinical benefits of MMDx have been successfully evaluated in a large number of clinical studies and for several diseases. The assessment of new diagnostic tests systematically reports conventional parameters, such as sensitivity, specificity, positive and negative predictive values, in comparison to a reference. In the absence of a more relevant reference for the evaluation of broad-spectrum molecular methods, MMDx clinical utility has been evaluated by analyzing its diagnostic added-value, specifically in culture-negative cases. In all reported studies, culture as the gold standard and reference for statistical analysis has been used, which MMDx proposes to complement. The molecular results have been evaluated for their clinical relevance prior to inclusion as part of reported added-value.
The next section demonstrates, based on peer-reviewed articles, such proven diagnostic added-value of MMDx in specific applications with a focus on Sepsis, Bone and Joint Infections, Infective Endocarditis, Bacterial Meningitis and Pyomyositis:

➢ *Sepsis*

Sepsis is a primary cause of death worldwide, in 2017, it affected about 48.9 million people and 11 million sepsis-related deaths were recorded globally [3]. Assuming that a significant proportion of sepsis patients initially receives broad-spectrum antibiotics, early species identification with MMDx offers the opportunity to (i) rapidly initiate targeted

therapy and thus (ii) improve patient outcomes. Further, the targeted treatment significantly limits unnecessary use of antibiotics and (iii) contributes to the fight against antibiotic resistance.

Fig. 3 highlights four independent studies [4, 5, 6, 7] evaluating the clinical performance of MMDx for the rapid diagnosis of bacteraemia. A total of 606 samples were analyzed and compared with culture, showing:

- Diagnostic sensitivity and specificity ranging from 66.7 to 100% and from 70 to 94.4%, respectively.
- Culture and MMDx positive concordant results were found in 31 to 47% of the cases.
- The clinical added-value of MMDx, expressed as rate of true infections identified in culture-negative samples, were 40 to 57%.
- Blood culture-positive and MMDx-negative results were obtained in 0 to 27% of cases, which may have been caused by a very low microbial load in the samples.

In complement to blood culture (BC), MMDx eventually allowed the identification of almost twice as many precisely identified infectious pathogens, with an average absolute added-value of 87% compared to cases identified by culture only, while providing results within one working day.

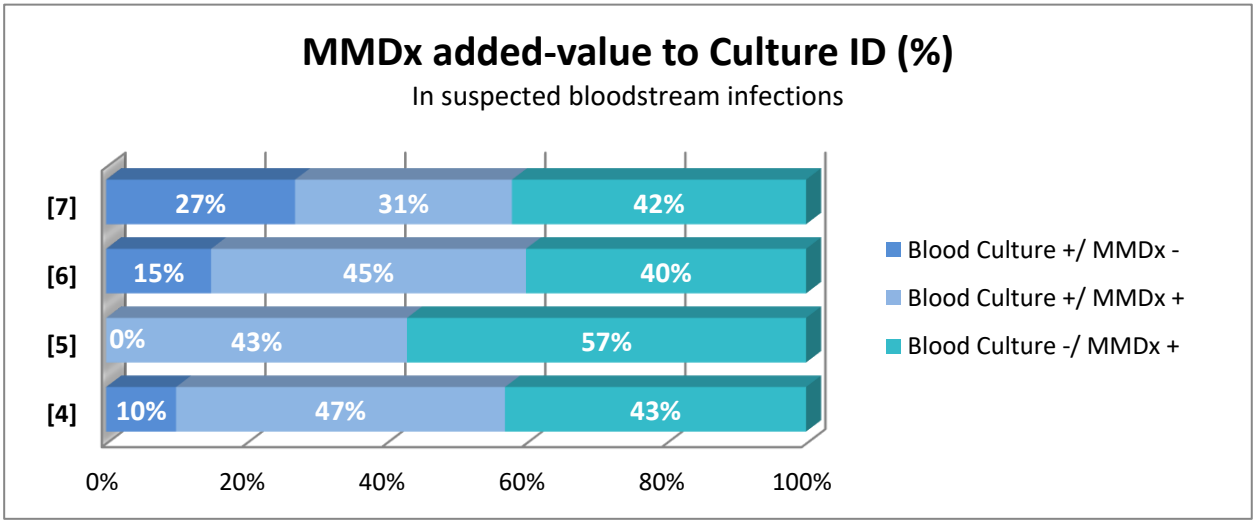

Fig. 3: Ratio of positive results by blood culture, Molzyms Molecular Diagnostic test (MMDx) or both methods obtained from whole blood samples; Combined positivity [4] 59/187 (32%), [5] 7/23 (30%), [6] 20/160 (12%), [7] 36/236 (15%)

➢ Bone & Joint Infections

In 2014, over one million total prosthetic surgeries were performed worldwide with an incidence of Prosthetic Joint Infections (PJI) ranging from 1 to 4% after primary knee replacement and 1 to 2% after primary hip replacement. Approximately 15—20% of all prostheses are found to be infected after primary revision surgeries. PJI with negative cultures poses a serious problem in regards to the proper diagnosis and patient management [8].

In a retrospective study, Marbjerg and colleagues [9] analyzed 529 samples – all of them culture-negative - from 223 patients. The specimens were processed using MMDx and the SelectNA™*plus* robot. The majority of the 378 specimens were from orthopaedic surgery, and the predominant sample type was tissue (n= 325), including mainly periprosthetic intraoperative tissue, but also bone and soft tissue. The number of MMDx-positive tissue samples was 105 (32.2%).

Furthermore, 85 samples of synovial fluids were analyzed, resulting in 25 (29.4%) positive

findings. In total, clinically relevant bacteria and fungi were detected in 181 (36.1%) positive samples. Antimicrobial treatment was adjusted in 42 cases (16.7%): in 30 episodes, antimicrobial treatment was changed according to the microorganism identified by MMDx and in 12 cases antimicrobial therapy was discontinued, following the MMDx-negative results.

The following table presents illustrative cases from Marbjerg et al., highlighting pathogen(s) identified by MMDx (in culture-negative samples only) that led to a change in therapy regimen, from inappropriate therapy to appropriate therapy, as determined by treating clinicians, in the context of orthopaedic samples.

Table 1: Pathogens identified by MMDx that led to a change in therapy regimen

Patient #	Specimen Type	Identified pathogen(s) (by MMDx; culture-negative)	Therapy before MMDx results	Therapy after MMDx results
1	Tissue	*Candida albicans*	Penicillin, Dicloxacillin	Fluconazole
2	Tissue	*Finegoldia spp., S. aureus*	Dicloxacillin, Penicillin, Clindamycin	Dicloxacillin, metronidazole
3	Synovial fluid	*Klebsiella spp., S. epidermidis*	Meropenem	Ciprofloxacin, vancomycin

➢ *Infective Endocarditis*

Infective Endocarditis (IE) is a relatively rare but life-threatening disease. In a systematic review of the global burden of IE, crude incidence ranged from 1.5 to 11.6 cases per 100,000 person-years and the contemporary mortality rate is approximately 25% [10].

In three studies [11, 12, 13] including a total of 186 dissected valve specimens from endocarditis patients according to Duke criteria, the diagnostic performances of MMDx and culture were compared. The diagnostic sensitivity of MMDx ranged from 61 to 87% and was 3 times higher than culture sensitivity, ranging from 20 to 87%. MMDx detected culture-negative pathogens or clarified discrepant culture results at considerable rates (26 to 67%). Fig. 4 displays the ratios of positive results of the two compared methods (for 2/3 studies gathering 140 specimens [11, 12].

The studies demonstrate that MMDx has a clear impact over valve culture in terms of i) significantly higher positivity rates, ii) discrimination of highly diverse multiple infections, and iii) identification of etiologies in culture-negative patients. Here, besides common also additional rare IE pathogens were observed.

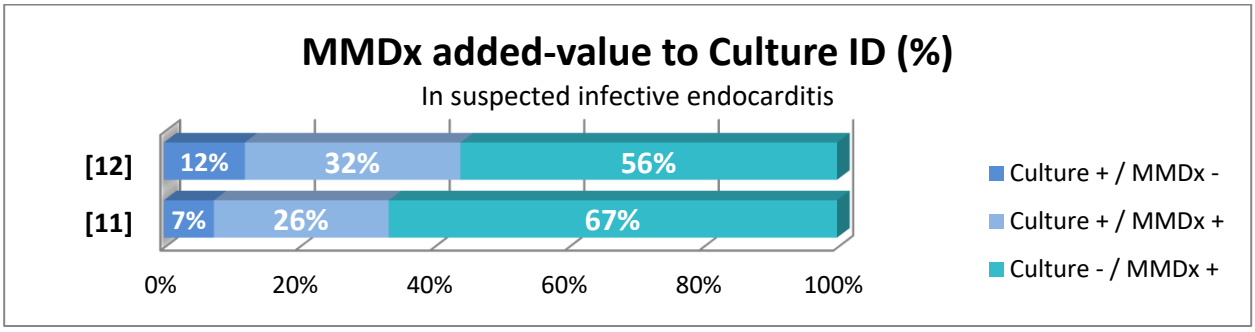

Fig. 4: Ratio of positive results by culture, Molzyms Molecular Diagnostic kits (MMDx) or both methods obtained from heart valves

➢ *Bacterial Meningitis*

Bacterial meningitis is a life-threatening disease associated with significant morbidity and mortality and requires immediate medical evaluation and treatment [14].
In a study from UKE Hamburg [15], 20 cerebrospinal fluid (CSF) samples from patients with suspected central nervous system infection, were analyzed with MMDx.
Pathogens causing Meningitis were detected in 13/20 samples (65%) compared to 7/20 (35%) by culture and/or microscopy, as shown on Fig. 5.
In another study in children [16], MMDx was able to detect the etiology of ventriculitis in 37/38 (97%) CSF samples, compared to 17/38 (45%) samples using culture methods.

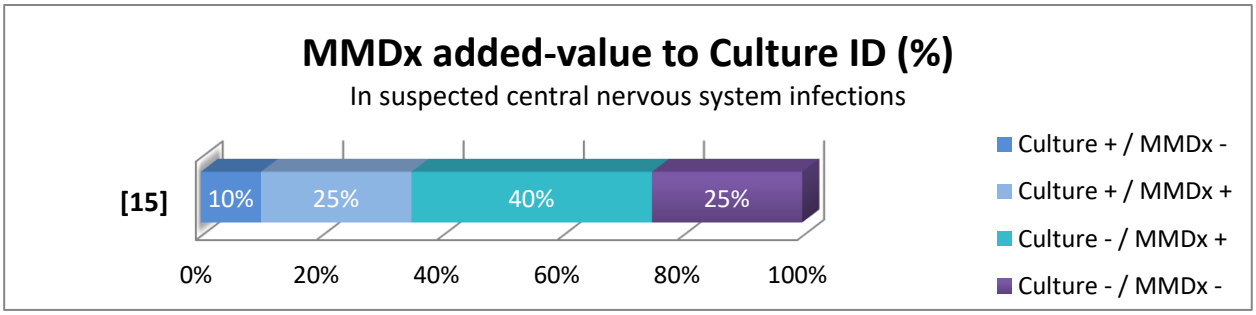

Fig. 5: Ratio of positive results by culture, Molzyms Molecular Diagnostic kits (MMDx), both or neither methods obtained from CSF samples.

➢ *Pyomyositis*

Pyomyositis is a rare, primary bacterial infection of skeletal muscle and diagnosis is in many cases challenging because most patients receive antibiotics before pus is collected from the muscle. In a recent study [17], samples from 12 patients were analyzed by culture and MMDx. At the time of pus aspiration, all patients were on antibiotic treatment. Fig. 6 illustrates that culture was positive in 5/12 (42%) cases and MMDx was positive in 12/12 cases (100%), all of them considered true positives. Antibiotic therapies were successfully switched from broad-spectrum to narrow-spectrum antibiotics, resulting in successful recovery of 11 patients. The study highlights the added-value of the accurate diagnosis of pyomyositis with MMDx, which identified an absolute value of 58% true infections in culture-negative results, in addition to the 42% concordant results obtained by both methods.

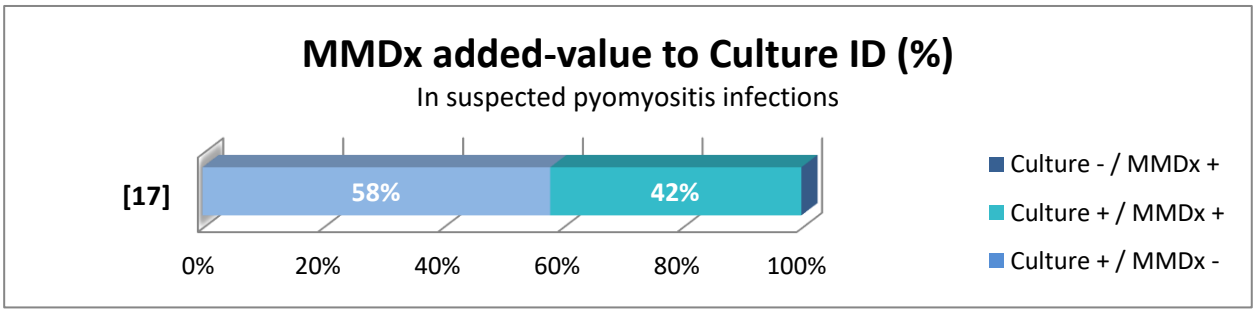

Fig. 6: Ratio of positive results by culture, Molzyms Molecular Diagnostic kits (MMDx) or both methods obtained from pus samples

Conclusion

All the reported studies showed that MMDx is a valuable method for the diagnosis of various infections, with the advantage of being culture-independent. A high concordance was reported between culture-positive and MMDx-positive results whereby MMDx displayed a high diagnostic added-value by identifying pathogens judged as clinically relevant in culture negative cases – by its capacity to detect non-viable/static or difficult-to-culture pathogens. It was also demonstrated that therapeutic decisions can be confidently made based on accurate identification of infectious agents by MMDx. Rapid pathogen identification allows earlier pathogen-adapted antimicrobial therapy in critically ill patients, reducing unnecessary use of broad-spectrum antibiotics, thus contributing to Antimicrobial Stewardship. The addition of MMDx, in complement to the traditional culture methods for microbial identification, would contribute in increasing the diagnostic positivity of laboratories that would benefit from a shorter time-to-result and considerable accuracy, to optimize the management of patients with serious infections.

Key Take-away

- ✓ **Various sterile sample types (body fluids, tissues, swabs)**
- ✓ **99% human DNA depletion & enrichment of microbial DNA**
- ✓ **Bacterial and fungal DNA detection by broad-range 16S and 18S PCR**
- ✓ **Identification on species level (> 1 300) by Sanger sequencing**
- ✓ **High concordance with culture positive results**
- ✓ **High positivity in culture-negative samples**
- ✓ **Clinical added-value in diagnosis of pathogens causing bloodstream infections, bone & joint infections, infective endocarditis, bacterial meningitis, pyomyositis etc.**
- ✓ **With the identification of (selection of organisms ID by MMDX):**

Staphylococcus aureus, Staphylococcus epidermidis, coagulase-negative Staphylococci, Streptococcus mitis, Streptococcus gallolyticus, Streptococcus pneumoniae, Streptococcus spp., Enterococcus faecalis, Enterococcus spp., Neisseria meningitides, Haemophilus influenza, Klebsiella pneumoniae, Escherichia coli, Corynebacterium spp., Clostridum spp, Parvimonas micra, Cutibacterium spp., Mycobacterium tuberculosis, Capnocytophaga canimorsus, Pseudomonas aeruginosa, Tropheryma whipplei, Coxiella burnetti, Bartonella spp., Granulicatella adiacens, Candida albicans, other Candida spp., etc.

Note

MMDx is cleared as CE IVD in Europe; it received Breakthrough Device Designation in April 2021 and is not yet available for diagnostic use in the United States of America.

References

[1] Ning, Y., Hu, R., Yao, G., Bo, S. (2016) Time to positivity of blood culture and its prognostic value in bloodstream infection. Eur J Clin Microbiol Inf Dis 35, 619–624. https://doi.org/10.1007/s10096-016-2580-5

[2] Lorenz M.G., Mühl H., Disqué C. (2015) Bacterial and Fungal DNA Extraction from Blood Samples: Manual Protocols. In: Mancini N. (eds) Sepsis. Methods in Molecular Biology (Methods and Protocols), vol 1237. https://doi.org/10.1007/978-1-4939-1776-1_11

[3] Rudd, K. E., Johnson, S. C., Agesa, K. M., Shackelford, K. A., Tsoi, D., Rhodes Kievlan, D., Colombara, D. V, Ikuta, K. S., Kissoon, N., Finfer, S., Fleischmann-Struzek, C., Machado, F. R., Reinhart, K. K., Rowan, K., Seymour, C. W., Watson, S., West, E., Marinho, F., Hay, S. I., … Naghavi, M. (2020). Global, regional, and national sepsis incidence and mortality, 1990-2017: analysis for the Global Burden of Disease Study. Articles 200 ww.Thelancet.Com, 395. https://doi.org/10.1016/S0140-6736(19)32989-7

[4] Wellinghausen, N., Kochem, A. J., Disqué, C., Mühl, H., Gebert, S., Winter, J., Matten, J., & Sakka, S. G. (2009). Diagnosis of bacteremia in whole-blood samples by use of a commercial universal 16S rRNA gene-based PCR and sequence analysis. Journal of Clinical Microbiology, 47(9), 2759–2765. https://doi.org/10.1128/JCM.00567-09

[5] Rogina, P., Skvarc, M., Stubljar, D., Kofol, R., & Kaasch, A. (2014). Diagnostic utility of broad range bacterial 16S rRNA gene PCR with degradation of human and free bacterial DNA in bloodstream infection is more sensitive than an in-house developed PCR without degradation of human and free bacterial DNA. Mediators of Inflammation, 2014. https://doi.org/10.1155/2014/108592

[6] Orszag, P., Disqué, C., Keim, S., Lorenz, M. G., Wiesner, O., Hadem, J., Stiesch, M., Haverich, A., & Kühna, C. (2014). Monitoring of patients supported by extracorporeal membrane oxygenation for systemic infections by broad-range rRNA gene PCR amplification and sequence analysis. Journal of Clinical Microbiology, 52(1), 307–311. https://doi.org/10.1128/JCM.02493-13

[7] Nieman, A. E., Savelkoul, P. H. M., Beishuizen, A., Henrich, B., Lamik, B., MacKenzie, C. R., Kindgen-Milles, D., Helmers, A., Diaz, C., Sakka, S. G., & Schade, R. P. (2016). A prospective multicenter evaluation of direct molecular detection of blood stream infection from a clinical perspective. BMC Infectious Diseases, 16(1). https://doi.org/10.1186/s12879-016-1646-4

[8] Parikh, M. S., & Antony, S. (2016). A comprehensive review of the diagnosis and management of prosthetic joint infections in the absence of positive cultures. Journal of Infection and Public Health, 9(5), 545–556. https://doi.org/10.1016/j.jiph.2015.12.001

[9] Marbjerg, L. H., Holzknecht, B. J., Dargis, R., Dessau, R. B., Nielsen, X. C., & Christensen, J. J. (2020). Commercial bacterial and fungal broad-range PCR (Micro-DxTM) used on culture-negative specimens from normally sterile sites: diagnostic value and implications for antimicrobial treatment. Diagnostic Microbiology and Infectious Disease, 97(2), 115028. https://doi.org/10.1016/j.diagmicrobio.2020.115028

[10] Holland, T. L., Baddour, L. M., Bayer, A. S., Hoen, B., Miro, J. M., & Fowler, V. G. (2016). Infective endocarditis. Nature Publishing Group, 2. https://doi.org/10.1038/nrdp.2016.59

[11] Kühn, C., Disqué, C., Mühl, H., Orszag, P., Stiesch, M., & Haverich, A. (2011). Evaluation of commercial universal rRNA gene PCR plus sequencing tests for identification of bacteria and fungi associated with infectious endocarditis. Journal of Clinical Microbiology, 49(8), 2919–2923. https://doi.org/10.1128/JCM.00830-11

[12] Peeters, B., Herijgers, P., Beuselinck, K., Verhaegen, J., Peetermans, W. E., Herregods, M.-C., Desmet, S., & Lagrou, K. (2017). Added diagnostic value and impact on antimicrobial therapy of 16S rRNA PCR

[13] Marsch, G., Orszag, P., Mashaqi, B., Kuehn, C., & Haverich, A. (2015). Antibiotic therapy following polymerase chain reaction diagnosis of infective endocarditis: A single centre experience. Interactive Cardiovascular and Thoracic Surgery, 20(5), 589–593. https://doi.org/10.1093/icvts/ivv006

[14] Brouwer, M. C., Thwaites, G. E., Tunkel, A. R., & Van De Beek, D. (2012). Dilemmas in the diagnosis of acute community-acquired bacterial meningitis. In The Lancet (Vol. 380, Issue 9854, pp. 1684–1692). Elsevier B.V. https://doi.org/10.1016/S0140-6736(12)61185-4

[15] Meyer, T., Franke, G., Polywka, S. K. A., Lütgehetmann, M., Gbadamosi, J., Magnus, T., & Aepfelbacher, M. (2014). Improved detection of bacterial central nervous system infections by use of a broad-range PCR assay. Journal of Clinical Microbiology, 52(5), 1751–1753. https://doi.org/10.1128/JCM.00469-14

[16] Stubljar, D., Kopitar, A. N., Groselj-Grenc, M., Suhadolc, K., Fabjan, T., & Skvarc, M. (2015). Diagnostic Accuracy of Presepsin (sCD14-ST) for Prediction of Bacterial Infection in Cerebrospinal Fluid Samples from Children with Suspected Bacterial Meningitis or Ventriculitis. https://doi.org/10.1128/JCM.03052-14

[17] Gabas, T., Podglajen, I., Cheminet, G., Gagnard, J. C., & Wyplosz, B. (2019). Diagnostic accuracy of 16S rDNA PCR in bacterial pyomyositis: A prospective study. Journal of Infection, 79(5), 462–470. https://doi.org/10.1016/j.jinf.2019.08.008