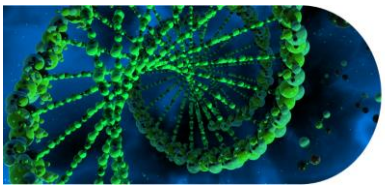

Application Note

Trends in Molecular Diagnostics No. 2/2021: 1-6

Broad-Range Molecular Detection of Rare and Fastidious Pathogens

Keywords: *routine molecular testing, in-vitro diagnosis, culture-negative infections, culture-independent, eubacteria, fungi, severe infections, tick-borne diseases, sepsis, endocarditis, joint infections, broad-range PCR, sequencing*

Marina Linow – Molzym GmbH & Co. KG, Bremen, Germany

Introduction

Rapid identification of infectious agents from patient samples is critical for the diagnosis and treatment of infectious diseases. Culture-independent diagnostic tests are increasingly used in clinical practice, as they offer several advantages over traditional culture methods and are now widely accepted as part of routine testing. First, they provide faster results – turn-around-time in hours rather than days. Further, they enable the detection of microorganisms that are difficult or even impossible to identify using conventional culture methods, with the capacity to detect microorganisms likely to be inhibited by antibiotics and for which routine laboratory tests are inefficient.

Broad-spectrum 16S and 18S rDNA PCR or Real-Time PCR and sequencing analysis is the ultimate method for accurate identification of pathogens. It especially offers the advantage of detecting the most common, but also difficult or impossible to culture bacteria and fungi from various sterile clinical specimen types, and has the capacity to cover polymicrobial infections. Compared with other singleplex or multiplex molecular methods that are limited to certain microorganisms or to panels of common pathogens, broad-range PCR is therefore particularly useful for the identification of difficult, rare, and low-incidence pathogens that are often associated with critical patient conditions that require rapid identification and appropriate antimicrobial therapy in a timely manner.

This application note emphasizes the clinical utility of Molzym's Molecular Diagnostic Systems (MMDx) for the accurate and culture-independent molecular detection of rare and fastidious pathogens - growing or static - and thus for the management of infectious diseases associated with them.

Molzym's Culture-independent Molecular Test System

Molzym's molecular diagnostic solutions SepsiTest™-UMD, UMD-SelectNA™ and Micro-Dx™ (referred to as MMDx) are designed for the *in-vitro* diagnosis of pathogens that cause sepsis, endocarditis, joint infections, peritonitis, meningitis, pyomyositis and other diseases. MMDx is based on a single protocol of human DNA depletion, microbial DNA enrichment and extraction (MolYsis™) directly from samples, followed by broad-range 16S and 18S rDNA PCR or Real-Time PCR and sequencing analysis. To date, more than 1,300 bacteria and fungi at genus or species level have been identified with MMDx in clinical trials, routine and other analyses. The complete list of microorganisms can be found here. The 3-step diagnostic workflow of MMDx is shown in Fig. 1.

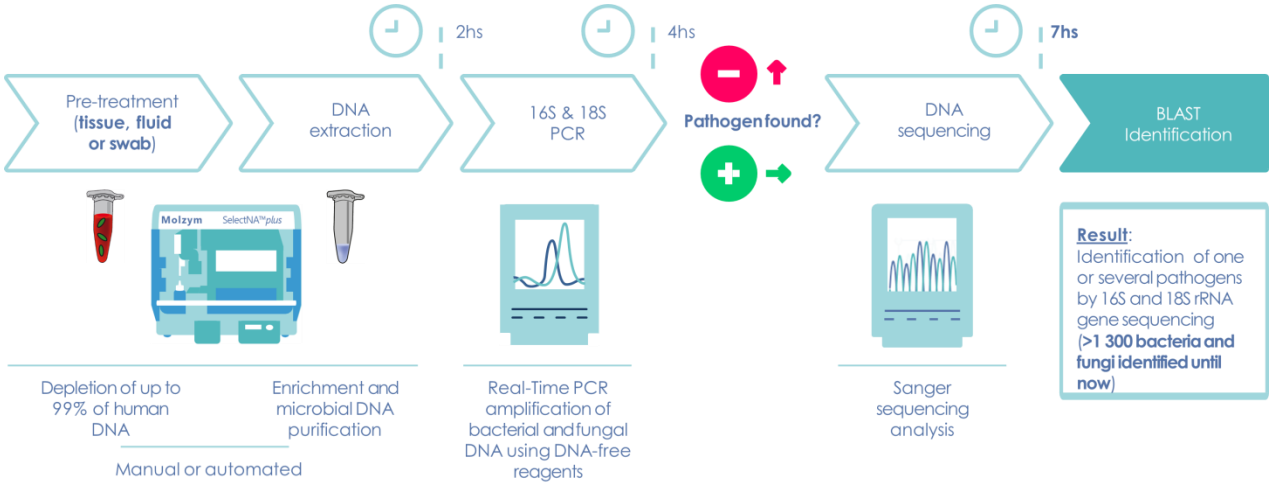

Fig. 1: MMDx broad-range diagnostic workflow

Detection of Rare and Fastidious Pathogens

The time to pathogen detection after specimen collection is crucial, especially in critically ill patients. Culture has long been the gold standard for detecting the causative agents of microbial infections. However, in some cases, cultivation fails to detect the pathogen [1], or cultivation takes several days because the microorganisms grow slowly. Fastidious microorganisms with special nutritional and environmental requirements may not grow, or sensitivity may be reduced if samples are collected immediately after antibiotic treatment [2]. Molecular techniques such as polymerase chain reaction (PCR) have introduced a new era in diagnostic microbiology. PCR using universal or specific primers and subsequent identification of the amplified product, mainly by sequencing, has enabled rapid identification of cultured and uncultured bacteria and fungi [1]. Hereafter, we present case reports and studies on the diagnosis of various culture-negative bacteria and fungi in which MMDx enabled the identification of e.g. *Candidatus* Neoehrlichia mikurensis, *Malassezia restricta*, *Bartonella* spp. and *Tropheryma whipplei* as well as other fastidious or anaerobic, slow-growing or non-cultivable microorganisms.

Candidatus Neoehrlichia mikurensis

In this case report conducted by UKSH Lübeck, Germany, MMDx results were compared to the standard of care (blood culture and specific PCR). This comparison shows how the result obtained by MMDx (Micro-Dx™ kit) has changed the clinical outcome [1].

History	A 48–year-old male was admitted with recurrent fever episodes up to 40°C for the last 4 months, weight loss > 10 kg, a general fatigue, overall reduced appearance. Earlier diagnosis: B-cell Lymphoma (three cycles of chemotherapy and splenectomy). Prior to admission, the patient was treated with piperacillin/tazobactam, meropenem and clindamycin without clinical improvement.
Lab results	Undulating white blood cells count, elevated C-reactive protein (CRP) and procalcitonin (PCT). Imaging: X-rays of chest and hip, MRI, TEE and a PET-CT scan, all without evidence of an infectious focus. Microbiology: Multiple sets of blood cultures = negative Molecular methods: EBV and Leishmania PCR = negative

Therapy	Empirically started on levofloxacin and prednisolone, patient discharged on oral levofloxacin after initial improvement, resumed shortly thereafter with fever > 40°C and chills. Therapy was switched to ceftriaxone without improvement.
Final Diagnosis	*Candidatus* Neoehrlichia mikurensis (CNM) was finally detected in a blood sample by applying broad-range 16S rDNA PCR (Micro-Dx™, Molzym) and sequence analysis for identification.
➔	Therapy was switched to oral doxycycline 200 mg with drastic improvement after 6 weeks of treatment; negative PCR control for CNM. A tick exposure was recalled retrospectively.

Candidatus Neoehrlichia mikurensis is a gram-negative intracellular pathogen that is transmitted by ticks in Europe and Asia [2] and was first identified as a human pathogen in 2010 [3]. CNM has not been cultivated to date and currently, the only diagnostic option is PCR [2].

Malassezia restricta

Blood culture-negative endocarditis (BCNE) diagnosis can be challenging. In a retrospective study the authors from University Hospital in Lyon, France, diagnosed 3 cases of *Malassezia restricta* BCNE from cardiac samples excised in a context of microbiologically nondocumented Infective endocarditis (NDIE) by using the highly sensitive MMDx (UMD-Universal kit) [4].
Out of 88 cardiac samples from patients who met the Duke criteria for definite infective endocarditis (IE), 16 were considered as NDIE-samples. Thirteen samples were 16S-PCR positive and 18S/26S/ITS-PCR negative, resulting in typical IE-causative bacteria such as *Streptococcus* (n = 9). Eight samples showed a polymicrobial etiology. The 3 other samples were found to be 16S-PCR negative but 18S-PCR positive with *Malassezia restricta* identified; results were confirmed using ITS- and 26S-PCRs. All 3 related patients had undergone cardiac valvular prosthesis implantation in the 2-15 months before excision surgery. Hospitalization was due to worsened general condition and moderate to severe heart failure with fever episodes. IE was proven based on the presence of inflammatory infiltrates (neutrophils) and fibrin layers in cardiac samples. Serological analyses did not show any cross-reaction with mannan-antigens but sera samples from 2 of the patients cross-reacted with *C. albicans* anti-mannan antibodies.
M. restricta appears to be an underestimated and underdiagnosed causative agent of NDIE, as it does not grow on routine culture media. Moreover, serological cross-reaction of *M. restricta* with *C. albicans* may lead to its misdiagnosis. This is of major concern since *M.* restricta is intrinsically resistant to echinocandins, the reference treatment for Candida IE [4].

Borrelia miyamotoi

In this case report, observed by a team at the Medical University of Vienna, Austria, a human infection with *Borrelia miyamotoi* is described [5].

History	A 51-year-old woman with a history of seropositive rheumatoid arthritis (treated with rituximab) was admitted with recurrent fever episodes that occurred over a period of three months. Four weeks before the onset of symptoms, the patient had been in the United States in several national parks, where she sustained several insect bites and a tick bite without erythema migrans. After her return, the patient stayed only in Lower Austria.
Lab results	No abnormal findings were observed on physical examination, no rash on the skin. Evidence of leukopenia and slightly elevated CRP. Microbiology: multiple blood and urine cultures = negative.

	Initial peripheral Giemsa-stained blood smear was negative, another one during the next fever episode was positive with spirochetes detected between blood cells. This could be related to prolonged spirochaemia due to the rituximab therapy.
Final diagnosis	EDTA blood was analyzed by MMDx (SepsiTest-UMD kit) and *Borrelia* spp. identified. *B. miyamotoi* was confirmed by specific PCR. Phylogenetic analysis of the *B. miyamotoi* strain clearly indicated infection with a strain from Europe.
Therapy	The patient was treated with 200 mg doxycycline once daily for 2 weeks and recovered successfully, no signs of recurrence were observed in the following 6 months.

B. miyamotoi is a gram-negative relapsing fever spirochete transmitted by ticks and belongs to the group of fastidious bacteria which are difficult to grow *in vitro*. Since 2011 many symptomatic *B. miyamotoi* infections in humans have been reported in Asia, North America, and Europe [6].

Diverse Rare and Fastidious Pathogens – Study 1

A retrospective study from Denmark by Marbjerg and colleagues evaluated MMDx (Micro-Dx™ kit) and its clinical value and impact on antimicrobial therapy decision [7]. The following study criteria applied: (i) specimens from normally sterile sites other than venous blood, (ii) culture-negative specimens, (iii) administration of antibiotics, and (iv) unexplained signs of clinical infection. A total of 529 specimens were analyzed, with 71.5% coming from orthopedic surgery departments. Predominant sample material was tissue (61.6%), but synovial fluid (16.1%), purulent aspirates (8.9%), pleural fluid (7.6%), CSF (5.7%), and BAL (0.1%) were also included in the analysis. Bacteria or fungi were detected in 36.1% of specimens and were considered clinically relevant in 31.5% of cases. The genera and species identified with MMDx broad-spectrum PCR reflect the distribution of sample types, with a predominance of samples from orthopedic surgery departments.

Table 1: Microorganisms identified by MMDx from 191 culture-negative samples; adapted from [7]

Species (n=227)	n (%)	Species	n (%)
Staphylococcus aureus	29 (12.8%)	***Capnocytophaga canimorsus***	5 (2.2%)
Staphylococcus epidermidis	43 (18.9%)	*Corynebacterium* species	8 (3.5%)
CoNS other than *S. epidermidis*	19 (8.4%)	*Cutibacterium acnes*	10 (4.4%)
Hemolytic streptococci (*S. dysgalactiae* and *S. agalactiae*)	10 (4.4%)	Anaerobic bacteria (incl. ***Fusobacterium necrophorum***, Finegoldia spp., Clostridium spp.)	24 (10.6%)
Anginosus group streptococci	13 (5.7%)	***Legionella* species**	2 (0.9%)
Nonhemolytic streptococci other than anginosus group	20 (8.9%)	***Borrelia species***	1 (0.4%)
Enterococcus species	4 (1.8%)	***Mycobacterium tuberculosis***	1 (0.4%)
Klebsiella species	2 (0.9%)	*Candida albicans*	4 (1.8%)
Haemophilius species	5 (2.2%)	Other of uncertain pathogenicity	25 (11.0%)
Neisseria meningitides	1 (0.4%)	*Granulicatella adiacens*	1 (0.4%)

A dominance of Gram-positive bacteria was expected in this setting and is consistent with the presence of *S. aureus*, CoNS, and various streptococci. Yet, more unusual

microorganisms have been recognized that can be difficult to culture, such as *F. necrophorum*, *Capnocytophaga canimorsus*, *Legionella species*, *Borrelia* species, and *Mycobacterium tuberculosis* (Table 1). Pathogens not normally covered by empiric treatment were also diagnosed, e.g., *Candida albicans* in joint infection.

In 16.7% of cases, an adjustment of antibiotic treatment was initiated based on the results with MMDx (Micro-Dx™ kit).

Diverse Rare and Fastidious Pathogens – Study 2

A team at the Medical University of Innsbruck, Austria, investigated the performance of MMDx (SepsiTest™-UMD kit) for rapid identification of pathogens in the diagnosis of bone and joint infections [8]. Eighty-three orthopedic specimens and 21 specimens from other normally sterile body sites were tested in parallel by culture and broad-spectrum PCR with MMDx for the detection of bacteria and fungi. Compared with culture, the diagnostic sensitivity and specificity of MMDx were 88.5% and 83.5%, respectively. The detection rate of MMDx (34.6%) was higher relative to bacterial culture (25.0%). In 13 culture-negative samples, pathogens have been identified by MMDx, including verified polymicrobial infections. In particular, anaerobic, fastidious, and non-cultivable organisms were identified, e.g., Clostridia bacterium, *Finegoldia magna*, *Granulicatella adiacens*, and *Tropheryma whipplei* [8].

Diverse Rare and Fastidious Pathogens – Study 3

In a prospective study at Motol University Hospital, Prague, Czech Republic, 1,370 samples of a wide range of specimens (heart valves, joint tissue samples, joint aspirates, whole blood samples, and culture-negative cerebrospinal fluid (CSF) samples) were analyzed with MMDx (UMD-SelectNA™ kit) [9]. MMDx results were compared with those of culture, and the additional diagnostic value of the MMDx approach in the case of negative culture was investigated. The additional benefit of MMDx was shown in 173 MMDx-positive specimens, particularly in heart valves and joint tissue samples. Broad-spectrum PCR proved to be extremely beneficial in the examination of heart valves and CSF. Compared to culture, it definitely helped clarify difficult-to-diagnose cases, including those in which patients were receiving antibiotic therapy at the time of sample collection or in case of fastidious bacteria or fungi (Table 2).

Table 2: Cases of high clinical relevance MMDx results in negative-culture; adapted from [9]

Clinical Material	Species	Clinical Material	Species
Heart valve	*Bartonella Quintana* *Staphylococcus lugdunensis* *Abiotrophia defectiva* *Streptococcus sanguinis*	Joint tissue	*Parvimonas micra* *Staphylococcus lugdunensis*
CSF	*Neisseria meningitidis* *Fusobacterium necrophorum* *Streptococcus agalactiae* *Streptococcus pneumoniae*	Blood DPHO (Dept of Paediatric Haematology and Oncology)	*Candida albicans* *Enterococcus cecorum* *Klebsiella pneumoniae*
Joint aspirate fluid	*Finegoldia magna* *Streptococcus dysgalactiae*	Blood ICU (Intensive Care Unit)	*Streptococcus pyogenes* *Fusobacterium necrophorum* *Neisseria meningitides*

Conclusion

The reported cases and studies clearly demonstrate that conventional culture methods have limitations in diagnosing difficult, anaerobic or uncultivable microorganisms. MMDx has proven to be a reliable and complementary method for accurate identification of such rare and difficult-to-culture bacteria and fungi in routine clinical practice thanks to its unique broad-range approach encompassing 16S and 18S rDNA PCR together with sequencing analysis. By providing contamination-free kits and reagents that enable processing of depleted human DNA preparations directly from a variety of clinical samples and amplification over 40 PCR cycles without background in negative PCR controls, Molzym greatly overcomes the challenges currently associated with molecular detection methods. With MMDx, rare and uncommon pathogens can be identified within hours, which often significantly improve the management of patients with severe infections by providing faster pathogen-adapted antimicrobial therapy.

References

[1] A. M. Hoffmann, S. Fetscher, J. Rupp, and S. Hauswaldt, "Recurrent fever episodes caused by Candidatus Neoehrlichia mikurensis in a patient with B-cell lymphoma and status post splenectomy," *ECCMID 2019, Poster Present. #1516*, no. Session PS084-Zoonoses: from animal to human, 2019.

[2] C. Wennerås, "Infections with the tick-borne bacterium Candidatus Neoehrlichia mikurensis," 2015, doi: 10.1016/j.cmi.2015.02.030.

[3] C. Welinder-Olsson, E. Kjellin, K. Vaht, S. Jacobsson, and C. Wennerås, "First Case of Human 'Candidatus Neoehrlichia mikurensis' Infection in a Febrile Patient with Chronic Lymphocytic Leukemia," *J. Clin. Microbiol.*, vol. 48, no. 5, pp. 1956–1959, 2010, doi: 10.1128/JCM.02423-09.

[4] L. Houhamdi-Hammou *et al.*, "Malassezia restricta: An Underdiagnosed Causative Agent of Blood Culture-Negative Infective Endocarditis," *Clin. Infect. Dis.*, May 2021, doi: 10.1093/CID/CIAB377.

[5] S. Tobudic *et al.*, "Human Borrelia miyamotoi Infection, Austria - Volume 26, Number 9—September 2020 - Emerging Infectious Diseases journal - CDC," *Emerg. Infect. Dis.*, vol. 26, no. 9, pp. 2201–2204, Sep. 2020, doi: 10.3201/EID2609.191501.

[6] K. Kubiak, M. Szczotko, and M. Dmitryjuk, "Borrelia miyamotoi—an emerging human tick-borne pathogen in europe," *Microorganisms*, vol. 9, no. 1, pp. 1–13, 2021, doi: 10.3390/microorganisms9010154.

[7] L. H. Marbjerg, B. J. Holzknecht, R. Dargis, R. B. Dessau, X. C. Nielsen, and J. J. Christensen, "Commercial bacterial and fungal broad-range PCR (Micro-Dx™) used on culture-negative specimens from normally sterile sites: diagnostic value and implications for antimicrobial treatment," *Diagn. Microbiol. Infect. Dis.*, vol. 97, no. 2, p. 115028, Jun. 2020, doi: 10.1016/j.diagmicrobio.2020.115028.

[8] K. Grif, I. Heller, W. M. Prodinger, K. Lechleitner, C. Lass-Flörl, and D. Orth, "Improvement of Detection of Bacterial Pathogens in Normally Sterile Body Sites with a Focus on Orthopedic Samples by Use of a Commercial 16S rRNA Broad-Range PCR and Sequence Analysis," 2012, doi: 10.1128/JCM.00362-12.

[9] J. Tkadlec *et al.*, "The use of broad-range bacterial PCR in the diagnosis of infectious diseases: a prospective cohort study," *Clin. Microbiol. Infect.*, vol. 25, no. 6, pp. 747–752, Jun. 2019, doi: 10.1016/j.cmi.2018.10.001.