MOLECULAR AND EXPERIMENTAL BIOLOGY IN MEDICINE

REVIEW ARTICLE

MOLECULAR DIAGNOSTICS IN THE CLINICAL MICROBIOLOGY LABORATORY: NEW DEVELOPMENTS

Sonja Obranic

Abstract: Molecular diagnostics is broadly available in clinical microbiology laboratories worldwide, especially for the detection and identification of difficult-to-cultivate microorganisms. The field of clinical microbiology has experienced significant changes over the past decade due to extensive molecular biology research that resulted in novel molecular diagnostics technologies. These new technologies are being introduced in clinical microbiology laboratories with the aim of improving sensitivity, specificity, accuracy and time-to-diagnosis, ensuring valuable data for effective infectious disease clinical management, infection control and surveillance. They have a potential to greatly improve general healthcare, but also present certain challenges, mainly regarding the cost and the proper definition of test ordering and interpretation. This review will discuss the current and potential application of next-generation sequencing, digital PCR and syndromic multiplex molecular assays in clinical microbiology.

Institute of Public Health of Varazdin County, Department of Medical Microbiology

Corresponding author:
Sonja Obranic
Institute of Public Health of Varazdin County, Department of Medical Microbiology, Varazdin, Croatia
e-mail: sonja.obranic@zzjzzv.hr

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INTRODUCTION

Clinical microbiology laboratories routinely detect and identify medically important microorganisms in order to manage clinical decisions as well as to monitor the spread of infectious diseases and antimicrobial resistance. The information obtained from a clinical microbiology laboratory is extremely valuable for proper patient management and infection monitoring and control. Therefore, the laboratory's priority is to produce results in a timely, sensitive and precise manner. Routine work in clinical microbiological laboratories traditionally includes the examination of phenotypic characteristics of microorganism cultures grown in ideal growth conditions. This approach is extensively being used in laboratories worldwide and is often supported by semiautomatic and automatic methods. However, this approach is still limited by its inability to fully characterize all the medically relevant microorganisms and to provide results in an appropriate time interval for clinical management.

The last two decades have been characterized by the rapid development of all aspects of technology, which has also reflected on significant developments in the field of molecular biology and biotechnology. New molecular biology techniques have become the basis for the development of various clinical microbiology laboratory techniques that allow a significant departure from the above-mentioned traditional techniques. New molecular microbiology diagnostic methods enable rapid, highly specific and sensitive microbiological diagnostics and present a major clinical advantage providing more complex and complete clinical information than classic methods.

Next-generation sequencing, digital PCR and syndromic multiplex molecular assays are modern methods in clinical microbiology laboratories that provide invaluable information that is directly usable in both patient management (such as timely and accurate diagnosis and administration of highly specific

antimicrobial drugs) and public health microbiology (such as antimicrobial resistance monitoring and control and infectious diseases outbreak investigations). This review will explore the current application of these methods and their potential for inclusion in the routine work of the clinical microbiology laboratory in future times.

DISCUSSION

Next-generation sequencing applications in clinical microbiology

Next-generation sequencing (NGS) represents technologically diverse methods that allow simultaneous and independent sequencing of a large number of DNA fragments. These technologies enable researchers to produce an enormous volume of sequencing data in a short period of time and at a relatively low cost, making them a useful clinical tool. NGS has successfully been used in clinical microbiology for a variety of applications, like whole genome sequencing (WGS) and metagenomic NGS.

Current applications of WGS include microorganism typing, epidemiology and outbreak investigations, antimicrobial susceptibility predictions and virulence factor determination. Metagenomic NGS, on the other hand, focuses on the identification of pathogens directly from clinical samples. This approach is very complex due to the polymicrobial sample content, but gives highly valuable information regarding the microorganism diversity of the sample.

WGS in epidemiology and outbreak management

Various evolutionary studies used NGS to examine the origin and spread of bacterial pathogens at a global level. Even though some of these evolutionary studies were exclusively research-oriented, their impact on clinical microbiology is crucial because they set the ground for clinical sequencing and generating reference genome databases. Several studies analyzed the epidemiology and global spread of various methicillin-resistant *S. aureus* (MRSA) strains. Harris et al.[1] used a high-throughput genomics approach to get a high-resolution insight into the epidemiology and microevolution of one of the widely disseminated MRSA clonal lineages, sequence type 239 (ST239). McAdam et al.[2] investigated the evolution and transmission patterns of a pandemic MRSA clone (ST36-II), collected from patients on three continents over a 53-year period. Authors used a high-resolution phylogenomic approach that provided them with an understanding of the emergence, transmission and hospital adaptation of a major MRSA clone. Monecke et al.[3] performed molecular typing of a pandemic MRSA strain (ST-239-MRSA-III) and investigated its intercontinental spread with the use of DNA microarrays and whole-genome sequencing. He et al.[4] used whole-genome sequencing and phylogenetic analysis for the investigation of the evolution and global spread of an epidemic *Clostridium difficile* strain (027/BI/NAP1). In their research, the authors managed to identify key genetic elements that enabled the rapid dissemination and spread of this epidemic strain through the global healthcare system. A study of Mutreja et al.[5] identified the phylogeny of the lineage responsible for the current cholera pandemic. The authors identified high-resolution genetic markers in a number of whole-genome sequences of *Vibrio cholerae* isolates and managed to explain the origin and transmission events that shaped the current pandemic.

Another WGS application comes from the field of public health microbiology and includes the investigation of outbreaks of pathogenic microorganisms both in hospital and community settings. Several retrospectively conducted studies used whole-genome sequencing data to determine outbreak patterns of infections caused by *E.* coli,[6] *M. tuberculosis*,[7] *Acinetobacter baumannii*[8] and the measles virus.[9] These studies had no impact on immediate clinical decisions; however, they demonstrated the valuable potential of using WGS in outbreak investigations. Studies that investigated outbreak events in real-time[10–12] provided detailed information in a clinically relevant time, which helped with the clinical management of outbreaks. These studies confirmed that WGS has great potential for enabling clinicians to take appropriate infection control and public health measures in a timely manner, thus reducing the impact of outbreak.

When considering the results of the aforementioned studies, one has to agree that whole-genome sequencing is a superior technique over the bacterial genotyping techniques that investigate only certain regions of the microbial genome and are historically used in microbial epidemiology studies and outbreak management. Particularly interesting is the ability to obtain information on microorganism transfer between health centers and even individual patients within the same healthcare facility. The amount and quality of information obtained by WGS goes beyond previously used techniques, although it is still uncertain when WGS will be introduced into the routine work of the clinical microbiology laboratory. The price of the methodology is one of its disadvantages, but there are other considerations like turnaround time, sequencing sensitivity, personnel requirements and tight quality control, that need to be addressed prior to WGS introduction into the routine laboratory workflow.

Antimicrobial susceptibility testing

A very exciting possibility of WGS application in clinical microbiology is the determination of antimicrobial susceptibility based on the identification of genes and/or chromosomal mutations that harbor

antimicrobial resistance. This methodology has the potential to significantly reduce the time to optimal antimicrobial therapy compared to conventional antimicrobial susceptibility testing (AST). Several studies published in recent years successfully used WGS for the determination of antimicrobial susceptibility of various pathogens such as *Staphylococcus aureus*,[13] *Mycobacterium tuberculosis*,[14] *Escherichia coli*,[15] *Neisseria gonorrhoeae*,[16] *Klebsiella pneumoniae*.[17] One recent study by Nguyen et al.[18] is of particular interest. The authors of this study used whole genome sequencing data together with paired antimicrobial susceptibility data of more than five thousand nontyphoidal *Salmonella* strains to generate a learning model for predicting minimum inhibitory concentration (MICs) for 15 antibiotics. This study is, to date, one of the largest published MIC modelling studies and bears a strategy for developing whole genome sequence-based models for surveillance and clinical diagnostics that have a high potential of application to other important human pathogens.

It is not very likely that complete replacement of routine phenotypic testing with WGS will be possible in the very near future; however, this new methodology does hold huge potential and it is a matter of time before the current limitations are overcome. Specifically, several issues need to be addressed in order for new technology to replace existing ones. Although the cost of sequencing has been significantly decreasing over time, the overall cost of setting up a sequencing laboratory is still a major obstacle for many laboratories. Furthermore, when looking from an expert perspective, a number of problems need to be solved in order to provide accurate, reliable, and clinically relevant antimicrobial susceptibility information from WGS data. Current antimicrobial resistance gene databases are very informative, but lack standardization and timely updating, which makes them insufficient for routine use in the prediction of antimicrobial susceptibility from whole genome sequencing data. In addition, complex mechanisms of antimicrobial resistance are multivariate, and often include multigene parameters, and/or different levels of transcription of individual resistance genes that determine the particular phenotype of antimicrobial resistance, which is difficult to recognize from the genome sequence alone. The development of machine learning algorithms that could accurately predict a clinically acceptable antimicrobial phenotype from an isolated genomic sequence, and which would be suitable for all clinically relevant microorganisms, could ultimately allow a complete transition from phenotypic testing to the determination of antimicrobial resistance via WGS.

Metagenomic NGS in clinical microbiology

Metagenomic NGS (mNGS) can be used in both untargeted and targeted approaches. In untargeted approaches, mNGS enables the characterization of all DNA or RNA found in a clinical specimen and represents an essentially hypothesis-free diagnostic approach regarding the causative agent of infection. Targeted mNGS uses specific PCR primers to enrich individual genes or genomic regions, which in turn increases the sensitivity for the detection of specific microorganisms, but decreases the potential to detect all microorganisms present in the clinical sample. Some of the major disadvantages of this method include high overall cost of test performance, questionable sensitivity due to the high host to pathogen nucleic acid ratio, the need for specific personnel not available to each laboratory and quality control concerns. However, some laboratories have been able to overcome many of the issues mentioned and successfully validated mNGS for the diagnosis of infectious diseases, such as sepsis[19] and meningitis/encephalitis,[20] demonstrating that the new methodology certainly has great potential for the use in clinical microbiology laboratories in the future.

Digital PCR - application for clinical microbiology

The term “digital” PCR was first used in the 1999 report by Vogelstein and Kinzler.[21] This method was, however, not new, as it had been established during the previous decade, and was known under the terms “single molecule PCR” or “limiting dilution PCR”.[22] Digital PCR (dPCR) is a method that determines the absolute concentration of nucleic acids, without the need for an external standard curve. In addition, it is more precise, more accurate in the presence of PCR inhibitors and offers more accurate quantitation in low amplification efficiency conditions when compared to quantitative PCR (qPCR). For all the reasons mentioned, dPCR has had various applications since its introduction, primarily in the fields of human genetics and oncology.

In the clinical microbiology setting, dPCR has several potential applications in the fields of virology, bacteriology and parasitology. There are several advantages of dPCR over more commonly used qPCR. One of them is the ability for the quantitation of pathogens that do not have well-characterized reference materials available. In addition, as dPCR if less affected by sequence variation, it is more appropriate for DNA quantitation than qPCR, when materials used for the standard curve and the sample show slight sequence diversity, as shown by Sedlak et al.[23] The authors of this study compared the accuracies of reverse transcription-quantitative PCR (RT-qPCR) and reverse transcription-digital PCR (RT-dPCR) for quantifying human rhinoviruses (HRV) RNA. Their results suggest that RT-dPCR is a method of choice for HRV quantification studies, as it more accurately quantified HRV RNAs across genotype groups that had up to two target-sequence mismatches within the primer or probe binding region.

Absolute quantitation of pathogen DNA performed by dPCR offers significant advantages over qPCR, as shown in several virus quantitation studies (CMV absolute quantitation,[24, 25] hepatitis E virus (HEV) RNA quantification,[26] hepatitis B virus DNA quantification,[27] HIV-2 plasma RNA quantification,[28] cell-associated HIV-1 RNA quantitation[29]). Due to the increased sensitivity over qPCR, dPCR may offer some novel applications, like detecting circulating human papillomavirus (HPV) DNA in patients with HPV-associated carcinomas. A study by Jeannot et al.[30] showed that in 87% of patients with invasive carcinoma (93% when using more optimally stored serum samples) HPV DNA was detected and quantified using digital droplet PCR (ddPCR, a variant of dPCR). DNA levels in cervical cancer patients were related to the clinical stage and the size of the tumor.

Besides virology, dPCR quantification assays can be used in other parts of the clinical microbiology laboratory, like parasitology and bacteriology. Two recent studies used ddPCR for sensitive and accurate quantification of human malaria parasites. Koepfli et al.[31] accurately diagnosed and quantified *Plasmodium falciparum* and *Plasmodium vivax* in clinical patients across parasite densities commonly observed in human blood. In addition, they showed that for low-density infections, quantification of malaria parasites by ddPCR yields more precise results than qPCR. Srisutham et al.[32] developed a new sensitive ddPCR assay for the detection and quantification of four human Plasmodium species.

In the field of bacteriology, digital PCR was recently used for the detection of *Mycobacterium tuberculosis* DNA from whole blood of patients with pulmonary and extrapulmonary tuberculosis in a study by Yang et al.[33] The authors of the study compared ddPCR and qPCR for detecting low levels of circulating *Mycobacterium tuberculosis* DNA. Their results showed an increased sensitivity for the detection of *M. tuberculosis* DNA with ddPCR over qPCR and concluded that ddPCR might potentially be used as a non-invasive, rapid and highly sensitive diagnostics tool for the detection of both pulmonary and extrapulmonary tuberculosis. In another study by King et al.[34] the authors used ddPCR for the detection and absolute quantification of *Borrelia burgdorferi* DNA in adult patients and *Ixodes scapularis* ticks. They concluded that ddPCR was as sensitive as a qPCR assay, but had some advantages over it, like fewer overall reactions and decreased sensitivity to PCR inhibitors.

The results of these studies suggest that dPCR might replace qPCR in a variety of clinical microbiology applications with respect to purpose, target microorganism and sample type. It is presumable that this methodology will be more readily used in clinical microbiology laboratories, especially after the optimization of several key factors such as test performance cost, laboratory workflow and quality control.

Syndromic panel-based testing of bloodstream infections

There are several US Food and Drug Administration (FDA) approved/cleared syndromic multiplex assays for rapid microbiology testing of positive blood culture bottles. Sepsis is considered a major cause of morbidity and mortality and also poses a healthcare economic burden, thus there is a great need to rapidly and accurately diagnose microorganisms that cause it.[35]

A number of clinical evaluation studies examined the characteristics of syndromic panel-based assays for rapid microbiology testing of bloodstream infections and their impact on clinical management. A retrospective study conducted by Ward et al.[36] tested two FDA-approved panel-based assays for the identification of both Gram-positive and Gram-negative bacteria and compared their accuracy, turnaround time for organism and resistance gene identification to standard clinical microbiology culture-based methods. Their results showed that both panels provided accurate results significantly faster than standard microbiology methods. However, in their setting, the earlier result had a modest impact upon clinical management. In the author's opinion, panel-based assays cannot be used as standalone tests, but rather as an add-on to conventional methods, due to their limitations in the choice of microorganisms and antimicrobial resistance genes as assay targets.

A study conducted by Mestas et al.[37] evaluated the use of a panel-based assay that identifies 12 Gram-positive organisms and three respective resistance markers for the diagnosis of bloodstream infections in pediatric population in a setting where the assay was performed 24 hours a day, seven days a week. The authors observed a dramatic improvement of the turnaround time, especially for the identification of methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant Enterococci (VRE).

A study by Ledeboer et al.[38] examined the use of a panel-based assays that detect eight genus or species targets (all Gram-negative) and six resistance determinants on a large number of samples (1847 in total). This study demonstrated high positive, PPA, (97.9%) and negative, NPA, (99.7%) percent agreements for bacterial identification targets in monomicrobial cultures when compared to standard microbiology methods and a PPA and NPA for the identification of six antimicrobial resistance genes of 98.3% and 99.9%, respectively. Authors conclude that the short assay result time and the valuable clinical information that it contains could have the potential to improve infection control, select appropriate antibiotic therapy earlier and reduce the overall cost of patient care.

Panel-based assays for the identification of bloodstream infection pathogens offer several advantages. They are simple to perform, require little time to set up, and deliver informative results in a short time, allowing early optimization of antimicrobial

therapy. Limitations of the method include the relatively high cost of test performance, especially since these tests cannot be used alone, but as an add-on to conventional methods for the diagnosis of bloodstream infections. In addition, it would be most clinically beneficial if these tests could be performed 24 hours a day, seven days a week; however, that might have a negative impact on laboratory resources for many clinical microbiology laboratories.

Syndromic panel-based testing of respiratory infections

A number of multiplex respiratory panels that simultaneously detect more than five pathogens have received FDA approval/clearance, and some more are available on market as CE-In Vitro Diagnostics (CE-IVD) assays. These assays differ in the number of pathogen targets included, time to result, and the number of samples they can process simultaneously. They offer sensitive and fast diagnostics compared to the conventional methods, such as viral culture, and have received clinical evaluation in a number of studies.[39-41]

The use of multiplex panel-based assay for the detection of respiratory pathogens may provide clinical benefits, especially when considering particularly sensitive populations, such as children or immunocompromised patients. Rapid diagnostics of respiratory infections caused by viruses reduces the unnecessary use of antibiotics, which ultimately results in the decrease of antimicrobial resistance and the overall cost of hospital treatment. One of the limitations of the method includes the inability to detect less common causes of respiratory infections. In addition, respiratory panel-based assays have a fixed combination of detectable microorganisms and cannot be tailored to the individual patient. Moreover, further clinical studies should investigate the clinical relevance of test sensitivity and detection limits for individual pathogens in the panel.

Syndromic panel-based testing of gastrointestinal infections

Standard microbiology diagnostics of gastrointestinal pathogens involves microscopy, culture, individual PCR assays and antigen detection methods. In recent times, a syndromic approach to the identification of gastrointestinal (GI) pathogens has become available with multiplex GI panel-based assays. There are currently several FDA-approved/cleared highly multiplex panel-based assays for the detection of GI pathogens. These assays differ in their time-to-result, hands-on time and the number of targets included in the panel. However, even the most time-consuming assays are significantly faster than stool culture (a few hours compared to 2-5 days). Several studies performed clinical evaluations of multiplex GI assays. Buss et al.[42] compared the use of a GI multiplex panel that detects 22 different enteric pathogens directly from stool samples with conventional culture testing and other molecular methods. This large study, performed on 1554 stool specimens, showed a high specificity and a sensitivity of 100% for 12 pathogens, and ≥94.5% for additional seven targets. The authors note that, due to the low number of positives, the sensitivity was not determined for the remaining three pathogens. The authors conclude that the assay offers improved performance over conventional microbiology methods and could aid in the reduction of infection transmission by directing appropriate therapy and infection control. Spina et al.[43] performed a multicenter study of community-acquired gastroenteritis comparing the use of a GI multiplex panel-based assay with standard laboratory procedures for the detection of GI pathogens. The authors detected at least one pathogen with the panel-based assays in 54.2% of the samples, compared to 18.1% found with conventional culture methods. Authors conclude with the remark that multiplex screening can dramatically improve the time-to-diagnosis and offer more clinically valuable information. Huang et al.[44] compared the performance of three GI panel-based assays for the detection of six common stool pathogens that were included in all off the assays. The authors were satisfied with the clinical performance of all assays in their patient population, and noted their advantage over conventional methods, such as the detection of a larger number of pathogens and rapid turnaround time.

Multiplex GI pathogen detection tests have several advantages over conventional microbiological testing. They enable rapid and accurate diagnosis of a variety of enteric pathogens in a single assay setup, enable small sample volume testing, increase the possibility of detecting coinfection, and show increased analytical sensitivity over conventional microbiological methods. Although the cost of the testing is high when compared to conventional methods, in many cases it is equal or even lower than the total cost of testing individual microorganisms represented in the multiplex panel. In addition, timely and accurate diagnostics provided with the use of GI multiplex panel has the potential to reduce the overall cost of hospital treatment, which favors the inclusion of these tests in the routine work of clinical microbiology laboratories.

Syndromic panel-based testing for central nervous system infections

Meningitis and encephalitis are very serious conditions that are associated with significant morbidity and mortality. Bacteria, viruses and fungi can all be causative agents of these conditions, with encephalitis having a more common viral etiology. First panel-based syndromic multiplex assay for CNS infections (FilmArray meningitis/encephalitis panel) became

FDA-cleared in 2015 and targeted 14 causative agents, with a turnaround time of approximately one hour directly from CSF specimens. Several studies evaluated the performance of this assay in clinical settings. Leber et al.[45] analyzed 1560 prospectively collected CSF samples with the multiplex panel assay and compared the results with standard culture (for bacterial analytes) and PCR (all other analytes). The authors concluded that the assay has overall good performance, with high sensitivity and specificity, and that it can serve as a great aid in the diagnostics of meningitis/encephalitis. Two studies evaluated this assay in the pediatric clinical setting. Messacar et al.[46] analyzed 138 samples from children with suspected central nervous system infections with the multiplex panel assay and compared the results with conventional diagnostics method results. Their results suggest that the multiplex panel-based assay can have comparable diagnostic yield and more rapid time-to-diagnosis when compared to conventional clinical testing of a suspected CNS infection in children. Graf et al.[47] performed a comparative evaluation of the FilmArray meningitis/encephalitis panel assay in 133 samples from pediatric population. Their study showed an overall agreement of 96.2% between comparator methods and the multiplex panel.

The results of these studies suggest that syndromic panel assays for meningitis/encephalitis have a comparable diagnostic outcome and allow a shorter time to diagnosis than standard microbiological techniques. Because a shorter diagnosis time for CNS infections may contribute to a timely clinical decision, it is presumable that its use will optimize the clinical outcome, reduce the use of unnecessary antimicrobial and antiviral medications, and shorten the length of hospital treatment. A considerable drawback for the introduction of syndromic diagnostic tests in routine clinical microbiology setting is the overall cost of laboratory equipment and reagents. A cost-benefit study for each clinical setting would be greatly beneficial before considering the introduction of these tests to routine laboratory workflow.

CONCLUSIONS

Next generation sequencing and its applications in whole genome sequencing and clinical metagenomics has great potential for becoming a routinely used technology in the clinical microbiology laboratory, for it can allow rapid identification and characterization of pathogens from a wide variety of samples. Regarding several matters, like cost, quality concordance, personnel qualifications, etc. it is not yet clear whether it could entirely replace routine microbiology procedures in the future. It is however apparent that in the times to come, more and more laboratories will begin to implement WGS according to their specific needs.

Digital PCR has considerable potential for the detection of pathogenic microorganisms in the clinical microbiology laboratories, due to its superior performance characteristics (in the terms of sensitivity, accuracy and reliability of experimental data) over routinely used qPCR. However, the cost of performing digital PCR, that is relatively high when compared with qPCR, might make its introduction into the routine work of clinical microbiology laboratories somewhat challenging.

Molecular multiplex panel-based assays have provided health care professionals with the opportunity to order a diagnostic test for the detection of a number of microorganisms associated with an infectious syndrome, instead of ordering a series of pathogen-specific individual assays. These assays significantly decrease the time-to-diagnosis period, which makes them a powerful tool for clinical management, including the use of appropriate antimicrobial therapy, infection prevention and control.

Since syndromic panel assays were introduced only recently, it is essential to establish clear algorithms and guidelines for their use and the interpretation of their results. With the further development of the technology, and the cost reduction of reagents and equipment needed for test performance, one can assume that many clinical microbiology laboratories will introduce these assays into their routine practice.

Novel technologies introduced in clinical microbiology laboratories will dramatically change the way infectious diseases are diagnosed, managed and monitored in the years to come. Even though it is unlikely that traditional microorganism culturing will completely disappear, it is reasonable to presume that novel technologies will take an important place in the routine work of clinical microbiology laboratories.

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