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Molecular Testing in Microbiology

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Abstract

There are significant challenges associated with qualitative and quantitative nucleic acid tests performed in diagnostic laboratories. The development of internationally available certified reference materials which can be traced to reference measurements will contribute to a better understanding of the performance characteristics of nucleic acid tests and enhance reliability and comparability of clinical data.

Next generation sequencing may have a future role in the identification and resistance detection of clinical pathogens, however, the current complexity of bioinformatics to support this technology makes its routine use in a diagnostic laboratory problematic. However, next generation sequencing is starting to impact epidemiological studies used to investigate the pathways of disease transmission in outbreaks and to determine microbial populations in metagenomics studies.

Keywords: Next generation sequencing; Metagenomics; Metrological traceability; Polymerase chain reaction

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Introduction

Many of the conventional microbiological methods such as culture and antigen detection are being replaced by molecular approaches which detect microbial nucleic acid by amplification methods or more recently with whole genome sequencing and analysis.

These new molecular diagnostic tests are being used for identification of pathogens, identification of antimicrobial resistance mechanisms in pathogens, quantitation of pathogens, determining the epidemiology of pathogen transmission either in whole populations (public health) or in special contained populations (infection control in hospital). However, there are currently a mixture of in-house and commercial nucleic acid tests with a wide variation in test chemistry and calibration standards with resulting variability in either qualitative or qualitative results. Currently most nucleic acid testing for microorganisms do not have standardised reference materials with traceability to SI and measurement procedures with the determined levels of uncertainty available. Exceptions to this are International Standards provided by the World Health organisation (WHO) for cytomegalovirus, human immunodeficiency virus, hepatitis C virus, Epstein Barr virus and Hepatitis B virus (1). These WHO virus standards are arbitrarily defined in international units with no independent reference (2)

Qualitative testing

Nucleic acid tests usually have greater sensitivity compared to traditional methods such as microscopy, antigen detection or culture as well as a more rapid turn-around time making these tests very useful in the clinical setting. Some nucleic acid tests can be used to determine drug resistance of microorganisms where specific gene mutations conferring resistance to an antimicrobial are known. However, as not all mutations leading to drug resistance are known, molecular tests do not replace phenotypic tests for determining microbial resistance in many situations.

Molecular testing for respiratory pathogens, particularly viruses, has significantly increased our ability to provide useful diagnostic information to clinicians in a timely manner for management of patients. Nucleic acid based testing for respirator

viruses offers much greater sensitivity, specificity and much shorter turnaround time than conventional methods (3, 4). As respiratory viruses, in particular influenza, cause significant morbidity, mortality and economic burden identifying the causative agent is important for epidemiological understanding of population based disease burden. Additionally, identifying a specific virus, for example influenza, may aid any decisions to commence antiviral therapy in high risk patients.

There are often very different levels of sensitivity of nucleic acid test between laboratories for detection of respiratory viruses. These differences may result from differences in nucleic acid extraction and amplification efficiency (5). Furthermore, when diagnostics are dependent upon RNA detection, the addition of the reverse transcription step can also offer significant variability depending on choice of reagents (6).

Quantitative assays

Quantitative assays for detection of microbial nucleic acids are mainly used for quantifying virus load where this information influences the clinical management of patients. Quantitative virus assays are performed in immunocompromised transplant patients mainly for cytomegalovirus virus (CMV), Epstein Barr virus (EBV), and BK virus. In patients undergoing therapy for chronic viral infections, human immunodeficiency virus (HIV), hepatitis B virus (HBV) and hepatitis C virus (HCV), quantitation of the viral load helps guide therapy.

It is essential that there is agreement in the quantitative nucleic acid tests where a specimen from a patient is repeatedly tested in the same laboratory and with results between different laboratories. There are many factors which contribute to the inter-laboratory variability in viral load quantitation. This variability may result from variability in reagents used during testing, variability in extraction methodology, the genes of the virus which are used for amplification and detection as well as the nature of the calibrator used to determine quantity. In addition, the use of different sample types, i.e. whole blood versus plasma, can also leads to variable results (1, 2, 6).

Further complicating the interpretation of quantitative levels of viral load, are the lack of a recognized and standardised inter-laboratory threshold for a viral load which would indicate the need to reduce immunosuppression or commence antiviral therapy. Currently it is strongly recommended that quantitative assays for viruses in patients for monitoring purposes are performed by the same laboratory to minimise variability. Recent results from our Quality Assurance Program in Australia for quantitation of cytomegalovirus gave inter-laboratory standard deviation ranging from 0.28 to 0.38 log10 copies per mL.

Molecular detection of nucleic acid is becoming more sensitive and can now detect very low levels of organisms making it increasingly important to determine cut-off values in organism load to determine whether the presence of the organism is causing disease or reflects patient colonisation.

Establishing metrological traceability

Development and implementation of an internationally recognized measurement system using internationally accepted reference materials with traceability will improve the ability to compare qualitative and quantitative nucleic acid test in microbiology across laboratories (2, 6). Recently the WHO developed international standards for CMV, EBV, HBV, HIV, HCV, BK virus (1). In addition the requirements for all testing laboratories to participate in an external quality assurance program to ensure agreement of methods and results with intra-laboratory and inter-laboratory assays will improve reliability.

It remains to be seen what impact the introduction of digital PCR, which can amplify a single molecule, will have on the quantitation of microbial nucleic acid. This technology uses nano-fluidics, a limit-dilution set-up with Poission statistics for analysis and does not require a calibration standard.

Next generation sequencing

Sequencing of nucleic acid has increased greatly in the last decade with the development of next (second)-generation sequencing (NGS) technologies largely replacing the original Sanger methodology. The much lower cost and higher throughput of NGS compared with Sanger sequencing has enabled implementation of this technology in many research and diagnostic laboratories. None of the current NGS technologies requires cloning of template DNA in to bacterial vectors, differentiating NGS from Sanger sequencing. With NGS there is a high degree of parallelisation, where millions of sequencing reactions take place at the same time in small reaction volumes, therefore, allowing a much higher throughput than Sanger sequencing.

Identifying Microorganisms

NGS has been used for identification of bacteria from culture colonies and also from pelleted material directly from specimens. The complexity of bioinformatics tools that assemble, analyse and interpret the genome sequence are major limitations of this technology for routine use at present. However, given the current costs of sequencing and

the complexity of the bioinformatics required this technology is unlikely to overtake matrix assisted laser desorption ionisation time of flight (MALDI TOF) mass spectrometry for organism identification in the near future. NGS may be of assistance with organisms which cannot be cultured or identified by conventional routine phenotypic means and which currently are identified using 16s rDNA sequencing. In a study of urine samples there was a high level of agreement between NGS and phenotype and predicted antimicrobial sensitivities for bacteria, indicating that this technology may be useful in the future (7).

Metagenomics

Analogous to the use of NGS methods to sequence multiple targets in a single organism is the ability to sequence and identify multiple organisms in a single specimen simultaneously - metagenomics. Metagenomics involves sequencing of DNA contents in a clinical sample before using bioinformatic analysis to filter out human and non-pathogenic organisms DNA to identify the causative agents. Metagenomics is used to characterise complex bacterial communities in environmental specimens or clinical specimens. For example, in sputum specimens from cystic fibrosis patients, metagenomics may allow detection of non-culturable fastidious organisms that may be out-completed and overlooked in routine cultures. However, a problem with this type of metagenomic study is the semi-quantitative nature of NGS which prevents an accurate assessment of the proportion of each organism present at a given time point or changes in the relative prevalence of microorganisms in serial specimens. Similarly, the presence of nucleic acid, does not necessarily indicate viable organisms but may represent residual nucleic acid from dead organisms or contaminated by exogenous sources of organisms entering the upper respiratory tract. Metagenomics remains experimental and studies of its uses and potential are now just emerging (8, 9).

Typing of Microorganisms

Another application for NGS is epidemiological investigation of outbreaks either within the community i.e. public health or within a hospital i.e. infection control. However, currently NGS is approximately twice the cost of traditional strain typing methods using pulse field gel electrophoresis (PFGE) or multi-locus sequence typing (MLST).

NGS can provide additional useful information including specific identification of resistance genes or point mutations associated with resistance to several classes of antimicrobials (10). Ultra deep sequencing, with multiple reads over the same genomic section, is designed to allow mutations to be detected at extremely low frequency in a population. Furthermore, as a result of the high number of parallel reads which provide highly redundant coverage of target sequences, NGS sequence data has very low error rates compared with Sanger sequencing (11, 12).

The main disadvantage of NGS technology is turn-around time which can be up to two weeks for a full genome including the need for significant computing bioinformatics expertise to assemble data. Due to the large amount of sequence data generated, these technologies are best suited to epidemiological studies rather than rapid identification of microorganisms or detection of single nucleotide polymorphism resulting in antimicrobial resistance in a clinical laboratory.

NGS generates large amounts of data which requires expertise in bioinformatics to assemble and analyse sequencing data using computer based algorithms. NGS may play a role with organisms that currently are unable to be identified using routine methods, including 16s rDNA sequencing or specific nucleic acid probes, to confirm identification of organisms that are not able to be readily cultured. Currently, there are numerous traditional typing methods including serotyping, binary typing, ribotyping, multi-locus variable-number tandem repeat analysis (MLVA), pulse field gel electrophoresis (PFGE) and multi locus sequence typing (MLST). These phenotypic methods have differing resolution power. NGS has the capacity to supersede many of these traditional typing methods due to *in silico* typing with superior discriminatory capacity.

NGS has been used to describe the evolution in epidemiology of important infections (13-16) and can be used to inform hospital infection control response to suspected pathogen transmission (17-20)

Virulence profiling

NGS may be used for detection of genetic marker of virulence such as panton-valentine leukocidine (PVL) in *Staphylococcus aureus* or shiga toxin in *Eschericia coli* (22). This information will require careful interpretation as regulation of gene expression may have a significant effect on phenotype.

Anti-microbial resistance detection

NGS can readily detect known mutations causing beta-lactam resistance and identify aminoglycoside modifying enzymes. However, NGS cannot reliably predict some resistance mechanisms resulting from alteration of regulatory genes or mutations in complex regulatory systems. While NGS may be able to predict anti-microbial resistance, the clinician needs to know anti-microbial susceptibility and it is

unclear how robust NGS will be in predicting susceptibility. NGS may be useful for slow growing organisms or organisms that are unable to be cultured or where phenotypic susceptibility testing is unreliable.

Conclusion

NGS is unlikely to overtake current methods for routine diagnostic bacterial identification and anti-microbial susceptibility testing in the near future. It will probably have an expanding role in reference, public health and infection control laboratories for detailed isolate characterisation, outbreak investigation and detection of disease transmission.

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