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Review

Exploring the challenges of molecular diagnostic techniques for clinical and veterinary microbiology in developing countries

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The advent of molecular biology techniques for the detection, diagnosis, monitoring and characterization of microorganisms have revolutionalized as well as increased the reliability of microbiology laboratory results. The fact that laboratories always served as an early warning system for epidemiologic surveillance, the use of molecular techniques also placed a huge challenge on microbiologists all over developing countries by leading the way into this new era by allowing rapid detection of microorganisms that were fastidious, previously difficult or impossible to detect by traditional (phenotypic) microbiological methods. Application of molecular techniques in clinical and veterinary microbiology laboratories have now progressed beyond identification of antibiotic resistance and tolerance genes, and are making inroads in the rapid and direct detection of etiologic agents of disease directly from clinical samples without the need for culture. Increased use of automation and user-friendly software makes these technologies more widely available, more efficient, less laborious, cost effective and gives room for versatile application. While the role of molecular techniques has increased in the developed world and are now part of routine specimen processing in many of these countries, it has continued to be a theoretical exercise in many developing countries and the evaluations of this technology for adoption in microbiology laboratories have generally been limited by several factors. As these molecular methods are further refined and become more widely available, microbiologists in developing countries will need to understand their clinical applications and be aware of their potential advantages, limitations and clinical utility.

Key words: Microbiology laboratory, molecular techniques, developing countries.

INTRODUCTION

Adequate clinical management of infectious diseases relies primarily on the accurate identification of the causal microorganism and the production of reliable information on its antimicrobial susceptibility (Foxman et al., 2005). Due to their clinical importance, misidentification of these pathogens in clinical samples can have serious consequences in increased morbidity and mortality. Over the past century, microbiologists have searched for more rapid and efficient means of microbial identification (Tang et al., 1997). The identification and differentiation of microorganisms had principally relied on microbial morphology and growth variables. These traditional methods of microbial identification rely solely on the phenotypic characteristics of the organism. Bacterial fermentation, fungal conidiogenesis, parasitic morphology, and viral cytopathic effects are a few phenotypic characteristics commonly used. Some phenotypic characteristics are sensitive enough for strain characterization; these include isoenzyme profiles, antibiotic susceptibility profiles, and chromatographic analysis of cellular fatty acids (Thurm

and Gericke, 1994; Pierson and Friedman, 1992; Lin et al., 1995; Stoakes et al., 1994; Blanc et al., 1994; Bernstein et al., 1994; Tenover et al., 1994; Asai et al., 1993). Traditional diagnostic methods in microbiology have limited the ability of laboratories to provide physicians with timely and clinically relevant information. Organisms may be in-correctly identified when the isolate shows unusual bio-chemical reactions or when the database does not include the correct identification. The short incubation time of susceptibility tests in some systems may cause problems. Bacteria with hetero-resistance to β-lactam drugs, inducible resistance mechanisms, or susceptibility gene mutation may be mis-classified. The resistance of pneumococci to penicillin, enterococci to glycopeptides, staphylococci resistance to oxacillin, and Enterobacteria-ceae to β-lactam drugs may be missed (Ferraro and Jorgensen, 1995; Krishner and Linscott, 1994; Tenover et al., 1995; Skulnick et al., 1992) and some will not grow on conventional media in the laboratory. However, recent technology provides results in minutes or hours rather than days or weeks (Table 1). In particular, molecular biological techniques have increased the speed and sensitivity of detection methods, as well as allowing la-zsboratories to identify organisms that do not grow or grow slowly in culture (Richardson and Smaill, 1998; Peruski and Peruski, 2003). These techniques also allow microbiologists to identify genes that result in resistance to antibiotics and to "fingerprint" individual isolates for epidemiological tracking (Pfaller and Herwaldt, 1997).

Molecular methods have become increasingly incurporated into the clinical and veterinary microbiology laboratories to diagnose diseases of both human and veterinary origins (Younis et al., 2000; Huff et al., 2000; Zadoks et al., 2002; Lee, 2003; Karahan and Cetinkaya, 2006; Speers, 2006). The tool of molecular biology have proven readily adaptable for use in clinical microbiology laboratory and promise to be extremely useful in diagnosis, therapy, epidemiologic investigations and infection control (Cormican and Pfaller, 1996; Pfaller, 2000; Pfaller, 2001) During the last decade there has been widespread application of techniques based on the amplification of specific nucleic acid sequences, and these have been applied increasingly for veterinary diagnostic uses (Pfeffer et al., 1995; Belak and Thoren, 2001; Leutenegger, 2001).

There is also a growing interest in the possibility of using molecular methods like PCR tests in the field or in mobile laboratories (Callahan et al., 2002). The main obstacles are the need for specialist equipment, technical difficulties in simplifying sample preparation and nucleic acid extraction, and considerations regarding quality control. This review focused on and overview some of the nucleic acid probe techniques that are available for use in the microbiology laboratory, evaluate the strength and weaknesses of these techniques look at reasons why many microbiologists in developing countries are lagging behind in the adoption and use of these technology with suggestions on the way forward.

TYPES OF MOLECULAR METHODS

Nucleic acid-based technology can be divided into hybridization systems and amplification systems, although most amplification technologies are also partly based on hybridization technology (Fluit et al., 2001). Nucleic acid probe hybridisation, the Polymerase chain reaction (PCR), the Ligase chain reaction, transcription mediated amplification, other evolving amplification methods, and nucleic acid sequencing form the basis of detecting and characterising an ever increasing range of viruses, bacteria, fungi, and protozoa (Table 2). This information is needed to type strains for infection control and other epidemiological purposes and to detect resistance genes or their surrogate markers.

Molecular detection has mostly come to the clinical microbiology laboratory in the form of Polymerase Chain Reaction (PCR) technology, initially involving single round or nested procedures with detection by gel electrophoresis. However, with the introduction of automation for the various stages of DNA or RNA extraction, amplification and product detection together with real-time PCR, molecular laboratories will continue to become more efficient and cost-effective (Henrickson, 2005). Microarray technology such as the DNA chip will likely further increase the utility of molecular detection in the clinical microbiology laboratory (Speers, 2006).

The variety of molecular techniques used for diagnostic applications demonstrate that no universal technique exists which is optimal for detection of nucleic acids. New techniques continue to be developed that involve a new approach to amplification, hybridization, formats, and labels (Kricka, 1999).

Factors limiting the availability and use of molecular methods in developing country laboratories

Inadequate skilled manpower

A 2002 estimate suggested that only 13% of the world's scientist live and work in Africa, Latin America or Middle East (Nchinda, 2002). In addition to the scarcity of experienced scientists, only very few teachers /lecturers in microbiology are conversant with the techniques and practical applications of molecular diagnostic methods in developing countries. Only those with such skilled know-

Table 1. Comparism of molecular and conventional microbiology methods.

Nucleic acid based techniques (Molecular technique)	Conventional (Phenotypic) microbiology techniques
Advantages	**Advantages**
1. Saves time, results can be in minutes or hours.	1. Some results can be obtained in minutes or hours (eg malarial parasite test)
2. Highly specific and highly sensitive.	2. Rise in antibody titer of serological procedure can suggest involvement of pathogen in infectious process.
3. Detects even fastidious or microorganisms that cannot be cultured at all on artificial media.	3. Can demonstrate viability of the target microorganism in culture.
4. Can still be used when antimicrobial agents are present or administered (that is, outcome of result is not affected by presence of antimicrobial agents in the patient or source of sample).	4. Phenotypic antimicrobial testing allows laboratories to test many microorganisms and can detect newly emerging as well as established resistance patterns. No prior knowledge of resistance gene is required.
5. Detection of certain properties such as toxin and other virulent genes or antimicrobial resistance.	5. Comparatively less expensive than molecular methods in terms of storage and use in developing countries.
6. Delay in sample collection and transportation has little or no effect on the final result.	6. Interpretation of results are usually not very difficult because they are not very complex.
7. Still effective when the microorganisms are nonviable or the viable number is low.	7. Few can still be effective when pathogen is dead (eg use of agglutination assays)
8. Quantification of microorganisms eg viral load in the blood.	8. Requires skilled labour. Though may not be as demanding as molecular methods
9. Can be used to pinpoint the source or origin of an epidemic pathogen	
Limitations	**Limitations**
1. Technically complex procedures. Though there are simple types, most requires highly skilled labour.	1. Difficult to use when organism is fastidious or cannot grow on artificial laboratory media.
2. High cost of initially establishing the laboratory.	2. Presence of antimicrobial agents affects many of the final result.
3. The accuracy, reproducibility and reliability depend on the technical expertise and experience of the scientist.	3. Delay in sample collection and transportation usually affects the quality of the final result.
4. DNA or RNA Detection cannot always demonstrate involvement in infectious process and cannot differentiate between viable and nonviable microorganisms.	4. Highly influenced by number of viable organisms and some are ineffective when the pathogen is dead.
5. Comparatively more expensive but their turnaround time can translate into an overall savings by providing a more timely diagnosis.	5. The accuracy, reproducibility and reliability depend on the technical expertise and experience of the scientist.
6. Due to their high specificity, may not detect newly emerging resistance mechanism and are unlikely to be useful detecting resistance genes in species where the gene has not been previously observed.	6. Less expensive, but delay in obtaining results could still lead to loses and wastages.
7. Potential for false-positive test results (e.g., by amplification of "contaminating" DNA). Poor primer design can also lead to erroneously positive results such that incidental amplification of microorganisms other than those sought occurs.	7. Potential for misidentification of pathogens that may exhibit atypical characteristics.
8. Potential for false-negative test results (e.g., because of presence of PCR inhibitors interfering with nucleic acid amplification).	8. Potential for false-negative results (e.g., when antibiotic are used before sample collection).
9. Also primers are designed based on the known sequences available through international databases but organisms or sequences yet to be discovered can subsequently reduce the specificity of the PCR (McHugh et al. 1995).	9. Most tests takes days to obtain a complete result.
10. Interpretation of positive molecular test results not yet	10. Many are not very specific and Variably sensitive.
	11. Comparatively costly in terms of time.

Table 1. Continued.

validated for all infectious diseases (e.g., latent vs. active infection). Viruses such as Epstein-Barr virus (EBV), Cytomegalovirus (CMV) and Herpes-simplex virus (HSV) are intermittently shed following primary infection without causing disease.	
11. Lack of Uniformity. Molecular diagnosis is also complicated by the vast array of in-house molecular tests used in different laboratories.	

*The table is not intended to be all-inclusive.

ledge can make the subject interesting and impact effectively.

Lack of will by individuals, policy makers and governments

The African Union (AU) leaders at the January 2007 Addis Ababa summit, proposed to enhance funding for scientific education and the environment as well as to set in place policies towards biotechnology development and intellectual property protection (Koenig, 2007; Masood, 2007). The AU leaders stopped short of approving a science and innovation fund and an independent assessment body for science on the continent. Lack of interest and constant display of nonchalance by our leaders, law makers and wealthy individuals who travel abroad for diagnostic research, medical treatments or to enjoy the products and services of other scientists abroad makes it difficult to acquire, transfer and adapt such tech-nology to developing countries (Ashok, 2005).

Environmental factors

In many developing countries, the establishment of specialized research institutes was intended to make research and some of these molecular techniques available to the local community, but lack of patronage by the community who may not have confidence on the local scientist have often been detrimental.

Cost of establishment and maintenance

Cost of most molecular methods is fairly high, this is because they require costly material and equipment. The sensitivity of the molecular methods makes the risk of contamination a very important issue in the establishment of laboratories for molecular techniques. To avoid false positive results due to laboratory contamination, relatively large laboratory areas are required for physical separation of reagent preparation, specimen preparation and product detection areas together with a high level of staff training and skill. Cost of purification, preservation etc can be discouraging (Pfaller, 2001; Foxman et al., 2005).

Poor scientific collaboration

Collaboration among scientists in developing countries is very poor and rare. Multi-center discourse needed to build collaborations is often accessed by conferencing with other scientists addressing similar problems (Okeke, 2007). A preference for developed country conference venues by scientists, unjustifiably high airfares from developing countries to the conference venues, difficulties in obtaining foreign visas, and limits in financial support for travel still hamper the participation of scientists from developing countries in international meetings.

Scarcity of relevant and up-to-date literatures

Significant roadblocks must also be overcome to bring the most up-to-date and relevant biomedical literature and electronic research resources to the desks of many scientists in the poorest countries. Many regional publications, which carry the bulk of papers authored by developing country scientists, have typically been neither free nor online.

Poor motivation from scientific societies and academia

Often, many scientific organizations and the academic societies believed the establishment and management of such specialized laboratories should be better funded by the government, unfortunately, the government may not be willing since the politicians are not knowledgeable and

Table 2. Some applications of molecular tools to various microbiology units.

Microbiology units	Diagnostic Method(s)	Microorganism(s)	Purpose of test /Comments	References
Clinical and Veterinary bacteriology	PFGE	Staphylococci	Epidemiological studies and genome analysis	Zadoks (2002) and Corrente et al. (2005).
	PFGE	Streptococci	Genome analysis and epidemiology	Chiou et al. (2004)
	PFGE	Leptospora	Genome analysis and epidemiology	Baril and Saint (1990)
	PFGE	*Chlamydia trachomatis*	Genome analysis and epidemiology	CDC (2002)
	Plasmid analysis	Staphylococci	Epidemiology and genome	Liu et al. (1996)
	Plasmid analysis	Enterobacteriaceae	Epidemiology and genome	Elbasha et al. (2000)
	Southern Blot	Salmonella	Epidemiology and detection	Bailey et al. (2002) and Singh et al. (2006)
	PCR	Staphylococci	Enterotoxin genes detection and pharmacogenomics.	Cockerill (2003), Paule et al (2003); Zanelli et al. (2004); Jenkins (2005)
	PCR	*Bordetella pertusssis*	Epidemiological survey and Pharmacogenomics,	Cockerill and Smith, (2004) and He et al. (2004).
	PCR	*Chlamydia trachomatis*	Detection of infectious agent in sexually transmitted cases	Toye et al. (1996) and Cook, et al. (2005)
	PCR	*Mycobacterium tuberculoses*	Detection of infectious agent	CDC (2003); Devulder et al. (2005)
	RT-PCR	*Bacillus anthracis*	Biosecurity, Detection of infectious agent.	Ivnitski et al. (2003)
	AFLP	*Mycobacterium avium* complex	Detection of infectious agent	Woods (2001)
	AFLP	*N. gonorrhoeae*	Pharmacogenomics and detection	Cook et al. (2005)
	PCR	*Tropheryma whipplei*	Detection and genome analysis	Dutly and Altwegg (2001); Fenollar and Raoult (2004)
	AFLP, PCR	*Helicobacter pylori*	Detection and genome analysis.	Colding et al. 1999
	AFLP	*Burkholderia* spp	Genome analysis and detection.	Coenye et al. (1999).
Clinical and Veterinary Virology	PCR	Severe acute respiratory syndrome (SARS-CoV)	Detection and genome analysis during an outbreak.	www.cdc.gov/sars/CD 03
	PCR	Cytomegalovirus	Pharmacogenomics, Viral load monitoring.	Emery et al. (2000) and Palesanthiran et al. (2002)
	PCR and Sequencing	Herpes Simplex virus (HSV).	Pharmacogenomics and Viral load monitoring.	Read et al. (1997) and Druce et al. (2000).
	PCR and Sequencing	Human Immunodeficiency Virus (HIV).	Pharmacogenomics, Viral detection and Viral load monitoring.	Seed et al. (2002), Berger et al. (2005) and Dax (2004).
	RT.-PCR	Hepatitis B virus	Viral load monitoring and pharmacogenomics	Dixon and Boehme (2000) and Thomson and Main, (2004).
	PCR	Hepatitis C virus	Detection and Viral load monitoring.	Dore et al. (1997), Fried et al. (2002) and Thomson and Main, (2004).
	RT-PCR	Newcastle disease virus	Detection of viral genome and Epidemiological survey.	Aldous and Alexander (2001).
	RT-PCR	Swine Vascular disease virus	Detection of virus and genome analysis.	Nunez, et al (1998); Zhang et al. (1999b).

Table 2. Continued

	RT-PCR	Blue Tongue virus	Viral detection and genome analysis.	Zhang et al. (1999a), Jonhson et al. (2000) and Zientara et al. (2002).
	PCR	Influenza Virus (H5N1, H1N1)	Viral detection and Viral genome characterisation.	WHO (2007); WHO (2008); Trifonove et al. (2009).
	PCR	Varicella zoster virus	Perinatal infection and viral diagnosis.	Heuchan and Isaacs (2002).
	Real time PCR	Adenovirus	Diagnosis of viral diarrhoea.	Clark and Mckendrick (2004).
Clinical and Veterinary Mycology	PCR	Pneumocystis jiroveci	Genome analysis and detection	Helweg-Larsen et al. (2002).
	PCR	Toxoplasma gondii	Detection and genome analysis	Gilbert, (2002)
	PCR	Cryptosporidium spp	Genome analysis and detection	Jiang et al. (2005)

Pulsed field gel electrophoresis (PFGE), Amplified fragment length polymorphism (AFLP), Arbitrarily primed PCR (AP-PCR), Real time PCR (RT-PCR).

may not be properly informed on the existence and importance of such diagnostic tools.

Very poor teaching facilities for students willing to work in molecular technology

Potential molecular diagnostic oriented pupils usually get frustrated as soon as they realized that their dreams of venturing into any molecular based research in developing countries cannot be realized, many transfer the ‘’not the “not possible ideology” to the younger students, and hence, the dreams and zeal of these students to study molecular based techniques are killed prematurely.

Investors apprehensive attitude

Since molecular technology is a new and constantly developing science, many potential investors in developing countries are apprehensive that the equipments and materials purchased for research and teaching can easily become obsolete and give way to newer and better facilities. Every investor wants to reap the benefit of his/her investment for reasonable time and the innovations in molecular technique is so fast that such time may not be realized.

Uncertainty and unanswered questions

As with all new technologies new questions arise which can limit the clinical utility of the test? For example how long should we expect DNA to persist after recovery or treatment and in what body fluids or tissues will they persist, how can we distinguish between contamination, colonisation and active infection, and is the detection of DNA from microorganisms from so-called sterile sites a normal variant?

Licenses, billing and reimbursement

Major obstacles to establishing a molecular diagnostics laboratory that are often not considered until late in the process are required licenses, existing and pending patents, test selection, and billing and reimbursement (Ferreira-Gonzalez and Garrett, 1996). Reimbursement issues are a major source of confusion, frustration, and inconsistency. Reimbursement by third party payers is confounded by lack of various Food and Drug Administration approval and Current Procedural Terminology (CPT) codes for many molecular tests. In general, molecular tests for infectious diseases have been more readily accepted for reimbursement; however, reimbursement is often on a case-by-case basis and may be slow and cumbersome. FDA approval of a test improves the likelihood that it will be reimbursed but does not ensure that the amount reimbursed will equal the cost of performing the test.

Fee-for-service

Perhaps more than other laboratory tests, molecular tests may be negatively affected by fee-

for-service managed-care contracts and across-the-board discounting of laboratory test fees. Such measures often result in reimbursement that is lower than the cost of providing the test (Ross 1999).

WAY FORWARD

Regional reference laboratories can be established by organized private individuals, organizations or communities and young microbiologists could be trained to manage such establishments to serve the host community as well as make monetary gains for the investors.

If the performance characteristics of these systems are found to be acceptable, the molecular diagnostic laboratory will be able to analyzed more samples with higher throughput in an economic fashion and require less highly trained personnel. This will allow the clinical microbiology laboratory to answer more questions routinely by molecular methods than just the detection and quantification of microorganisms (Speers, 2006).

Not all molecular diagnostic tests are extremely expensive. Direct costs vary widely, depending on the test's complexity and sophistication. Inexpensive molecular tests are generally kit based and use methods that require little instrumentation or technologist experience. Low-cost molecular kits can be acquired by institutions, hospitals and other stake holders. This will help in giving the microbiology oriented students a firsthand practical knowledge of the application of these diagnostic tools.

Massive sensitization program and awareness on the importance and availability of these tools should be forwarded to the policy makers in various developing countries. This will make them know that instead of traveling to developed countries, these technologies can be acquired and transferred to their communities to make it accessible for the poor.

Microbiologists in developing countries should not just sit and wait for their governments to do things but rather see it as a challenge to develop and update their knowledge and skills. This will make them more relevant and make them compete favourably with their colleagues in other parts of the world.

Subsidized tests can be made available by government and charity organization in health institutions or reference laboratories. This will encourage the local communities to patronize and actually enjoy the benefit of these molecular tools; this is because people shy away from expensive laboratory tests in developing countries irrespective of their importance.

Local scientists especially microbiologists should renew their efforts in research directed towards adopting these molecular tools and modifying or inventing newer home-grown methods that are also sensitive, specific, cost effective and has the attributes of a good typing technique. We can do it, if the will is there! Equally, more effort is needed to promote and enable developing country scientists to study high impact problems, to define and manage their own research agenda, and to develop like-minded research nexuses (InterAcademy Council, 2004; Aldhous et al., 2005)

There is need to ensure sufficient and meaningful representation and participation of public and private institutions and researchers from developing countries including providing developing countries (governments and civil society) an equal voice in decision-making processes. Such participation will strengthen and build research and local production capacity of developing countries, and also ensure effective mechanisms/ measures to promote transfer of technology to developing countries.

Indigenous and foreign scientists must ensure that efforts are focused on the development of products that are adapted to the needs of developing countries, and the needs of patients of all ages, simple (in terms of using, prescription and storage), accessible (in terms of availability and affordability) and of quality. They should also ensure that prices of products/technologies produced are fixed with the aim of achieving equitable access to products/technologies to all that need those products/technologies in developing countries including middle income countries.

A collaboration between Microbiology societies and other scientific organizations in developed and developing countries should be facilitated to enable them work together and to ensure that outcomes and data generated from their researches are not monopolised but are shared in the public domain, widely disseminated for other researchers to engage in additional or follow-on health research.

Workshops, seminars and training can be organized by Microbiology Societies of developing countries to train and update their members on the latest technologies and their applications. This will make the acquisition of the knowledge cheaper, and create a conducive atmosphere for learning.

CONCLUSION

It is obvious that there is an urgent need for mechanisms for coordination, prioritization and ensuring adequate financing from the prices of the product; ensures availability of diagnosis and treatments that are suitable for developing country conditions and that are affordable; promotes generic competition as well as strengthens production capacity of developing countries.

It is equally important that the scientists in developing world now begin to conceptualize new diagnostic ideas that are reliable, effective, sensitive, sustainable and affordable to the people. Molecular methods are very expensive; this is a strong signal that issues of access must not be seen separately from issues of innovation. This is particularly important as the present system of innovation from developed countries is based on an incentive system (that is, the patent system) that depends on high prices to recoup investments, which then often results in the very innovative diagnostic methods being unaffordable to patients and the researchers that need them the most. Thus any model for developing countries must also consider the issue of "access", that is, how to make available appropriate and affordable technologies to developing countries.

Modern microbiologists in developing countries are very fortunate to have a variety of tools which provide good molecular identification of pathogens and which can be tailored to fit the needs of both the veterinary laboratory and the clinical studies. Opportunities to develop a personal career in molecular diagnostic methods and possible establishment of such laboratories are worth looking into. Many pathogens are not identified or misidentified and developing countries are like virgin area of research, but the introduction of molecular tools might possibly lead to discovery of previously unknown microorganisms and better understanding of the known microbial populations within these environments.

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