Applied Microbiology: Open Access
ISSN: 2471-9315

Bajinka and Secka, Appli Micro Open Access 2017, 3:1
DOI: 10.4172/2471-9315.1000130

Commentary Open Access

Integration of Molecular Methods into Microbiological Diagnostics

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Received date: February 21, 2017; **Accepted date:** March 07, 2017; **Published date** March 17, 2017

Abstract

Conventional microbiological methods take a long time to complete and sometimes accuracy can be compromised due to varying levels of expertise in the laboratory. Thus, molecular methods are highly needed to accomplish this mission. Apart from the specificity and sensitivity, molecular methods confer accuracy, precision, reproductive among others.

This review points out the respective needs for molecular techniques in diagnostic laboratories. Various convincing points were elaborated including; the short turnaround time, minimization of nosocomial infections (transmission within the community and health care system), the economic cost involved in-patient treatment, the sensitivity, reliability and the accurate diagnosis of infectious diseases.

The benefits that epidemiological studies draw from molecular techniques need to be implemented in developing nations, the big hopes, some limitations and recommendations of the use molecular methods in microbiological diagnostics are discussed.

Keywords: Infectious diseases; Molecular methods; Nosocomial infection; Microbial diagnosis

Introduction

The need for molecular methods in the microbiological diagnostics of infectious diseases has come of age. The reasons are based on; the timely advantages, detection of slow growing pathogen or nonculturable organisms that are more or less difficult to detect, identify and even to be test for antimicrobial susceptibility by conventional methods [1-5]. This century is conciding with many highly infectious pathogens, notably HIV AIDS that has been dictating our immune response to the emerging of number of infections [2,3,6-10].

	Culture	Molecualr
Sensitivity	Fair	3 to 5 fold greater than culture
Specificity	Very High	Very High
Vulnerability to transport promblems	Sensitive to extreme temperatures and drying	Viable specimen not required
Typing	2nd step often required	Incorporated in initial Procedure
Turn around Time	2-3 days	4-8 h

Table 1: Comparing culture and molecular testing (Felix Martinez, Jr., M.D Genital Herpes: Molecular Testing Can Increase Sensitivity For Detection of Herpes Virus, 2012).

Nowadays conditions such as HIV depends entirely on molecular diagnosis for susceptibility testing and management even during the acute phase [11]. In fact the hurdles that are faced when diagnosing a plethora of respiratory pathogens can now be accurately achieved even in a simpler and quicker manner than that offered by conventional approaches [1,10]. In the recent past, the replacement of traditional methods by molecular methods was thought to be far from achievable but with an increase in variety of commercially available kits, these hopes have now been reinstated [12]. Table 1 that shows molecular testing winning the upper hand against the culture method.

Sensitivity of the Tests

Humans are always prone to making mistakes and these ranges from fictions to realities. However, with the invention of more sophisticated machines a considerable reduction in human error have been assured. From conventional diagnostic to molecular methods, conventional PCR to real time PCR, from singleplex to multiplex PCR and from this stage to the promising high-throughput sequencing (HTS). With all of these, the purpose of achieving one condition will never be defeated; the sensitivity that molecular techniques has to offer. With the advance of techniques like; microfluidics, mutliplex with its Taqman probe system and information processing, sensitivity will always stands despensable (Table 2) [13].

Apart from the early detection of Mycobacterium tuberculosis, Chlamydia trachomatis, meningo-encephalitis syndrome and respiratory infections, the use of molecular methods also confer accurate diagnosis [1,3]. In addition to the different PCR's techniques, sanger sequencing, pryosequencing, mass spectrometry, microarray analysis and reverse hybridization, the molecular diagnosis has established an advanced blood culture techniques for identification and antimicrobial susceptibility tests (Antimicrobial susceptibility tests are commonly used to determine which specific antibiotic is sensitive to a pathogen). Nowadays a specific diagnosis and a concret decision

can be for specific treatment options in the mangament of infectious diseases (Table 3) [2,13].

Technique	Sensitivity	Specificity	Advantages	Disavantages
Culture Methods	50%	100%	Gold standard: Allows susceptabilities	Delay in Diagnosis: Low sensitivity
PCR Methods	90%	100%	Highest accuracy	Low commercial availability
ß-D-glucan	70-100%	87-96%	Panfungal marker: high sensitivity and specificity	many false-positive results;methodological concerns
Galactomannan	80-90%	80-90%	Increased accuracy for detection of Aspergillus in hematologic illnesses	Only for Aspergillus;many false-positive results; not useful in hematologic illnesses
Mannan or Antimannan	60-89%	80-84%	Good specificity and sensitivity with combined use	Results vary: limited experience
combination	87%	84%		

Table 2: Accuracy of diagnostic testing for invasive fungal infectionsin the ICU.

Inoculum Shigella	Multiplex PCR Positive results		Culture Positive results
(CFU/Sample)	Enriched PCR Samples	Direct PCR samples	
107	3/3	0/3	10/10
106	3/3	0/3	5/10
105	10/10	0/3	1/10
104	10/10	0/3	0/3
103	0/10	0/3	0/3
102	0/3	0/3	0/3
10	0/3	0/3	0/3
Non Inoculated	0/10	0/3	0/10

Table 3: Detection level of the PCR assay (Direct and Enriched) ascompared to the conventional culture, using serial dilutions of Shigella flexneri spiked to lettuce samples.

Reliability of the Results

Molecular techniques has added an exceptional discriminating power that confers a more reliable identification among and between strains of pathogens with high accuracy [1.16.5]. Of course, we will have to resort to methods that are more sophisticated if traditional methods failed to produce a good result. The low sensitivity arising from microscopy for example protozoan like; Trichomonas vaginalis, Neisseria gonorrhoeae (sexual transmitted infections) and intracellular pathogens like; virus, *Mycoplasma genitalium* not neglecting the low sensitivity of *Chlamydia* sp. and *Neisseria* [2] has caused for an alarm that the application of more inovative and sensitive methods is highly needed. In addition, other challenging calls for concern are the seropositivity (the quality or state of being seropositive, of having blood serum that tests positive for given pathogen) in *Chlamydia* sp. and the very slow growth of *Mycobacterium tuberculosis* also catalazed the need for a more sensitive techniques [2,14]. In a paper on 'Is There a Fungus Among Us - An Update on Diagnostic Strategies', PCR methods shows an increased 90% sensitivity while 50% for culture methods. This automaticaly catalyst the need for molecular methods to replace the less sensitive culture testings.

Detection and Identification of Nosocomial infections

The use of molecular methods will also reduce the risk of transmission within the community and health care system nosocomial infections [1,4]. Apart from its health complications, nosocomial infections also cause an increase in the cost of intensive care and prolong hospitalization by weeks or more [4]. Although with conventional methods, the study of epidemiology on nosocomial infections is next to impossible, with the invention of the molecular methods of strain typing; a positive difference have been seen [4,14]. The introduction of molecular biology methods that can detect DNA and RNA (nucleic acid probing and amplification) will no doubt help in strain typing for the types of nosocomial occuring at a particular time [1,6].

In Epidemiological Studies

Epidemiological studies are important to understand the transmission mechanisms and the role of microbial variants in the spread of diseases in society [15]. In the past decades, epidemiological studies were based on only phenotypical identification and for this, strain typing is not always possible [1]. The need for reference-based on data in the diagnosis has been highlighted through the use of nucleic acid amplification techniques [4]. And this has given a greater room for improvement in scientific discoveries [1]. Understanding the antimicrobial susceptility profiles for an organism is an important first step for studying epidemiology [7,15].

Improved Laboratory Turn-around Time (TAT)

The detection and identification of pathogens in a clinical laboratory is traditionally done by the microscopy, culture and sensitivity (MC&S).The sample can be view under microscope as the first stem that can quickly enable the clinician to decide on the commencement of the treatment based on the gram type. This is normally followed by culturing of the pathogen that passes through sub cultural and hence colony count is possible. Moreover, a pure culture is then subjected to a disc contining agar with antibiotics that can show the resistant and sensitive pathogens. These proceudures are loborious and time consuming [1.10.14] However when pathogen(s) can be detected in few hours after a patient visit the hospital, there is always an improvement and patient care is ensured [1,6,16,17]. Culture based methods can take days and sometimes weeks before the discrimination of sample [1,12]. And, to ensure pure isolation, sub culture causes further delays [10]. In order to create a boundary for the

morbidity and mortality rate that Invasive Fungal Infections (IFIs) are causing, early and acurate diagnosis is the key [8,9]. The clinical decisions made due to results obtained from the use of conventional methods, histological and radiological techniques in mycology are less rapid, sensitive and accurate when compared to molecular techniques (Table 4) [8]. Molecular methods can quickly establish antifungal therapy and avoid the toxic production by the antifungal agents in a more reliable manner [8,9].

Detection Method	Food borne Pathogens	Detection limit	Food matrix	Assay time
Multiplex PCR	*Salmonella spp., Salmonella enteritidis*	10^3 CFU/mL	Artificially and naturally contaminated chicken carcasses, minas cheese and fresh pork sausages	24 h
	STEC O26, O103, O111, O145, sorbitol fermenting O157, non-sorbitol fermenting O157	5×10^4 CFU/mL in minced beef and sprouted seeds. 5×10^3 CFU/mL in raw milk cheese	Artcificailly contaminated minced beef, sprouted seeds (soy, alfafa and leek) and raw milk cheese	24 h
	Escherichia coli O157: H7, *Listeria monocytogenes*, *Staphylococcus aureus*, *Yersinia enterocolitica*, *Salmonella*	10^3 CFU/ mL	Artificially contaminated pork	Not stated
Real-time PCR	*Salmonella enterica*	41.2 fg/PCR for Salmonella typhimurium genomic DNA, 18.6 fg/PCR for *Salmonella enteritidis* genomic DNA	Artificially contaminated chicken, liquid egg, and peanut butter	10 h
	Listeria monocytogenes, *Escherichia coli* O157, *Salmonella spp*	<18 CFU/10 g	Artificially contaminated ground beef, Naturally contaminated beef, pork, turkey and chicken	24 h
	Listeria monocytogenes, *Escherichia coli* O157, *Salmonella spp*	2×10^2 CFU/mL	Artificially contaminated ground pork	24 h
	Salmonella spp., Listeria monocytogenes	5 CFU/25 g	Artificially and naturally contaminated meat, fish, fruits, vegetables, dairy products, eggs, chocolate bar, omelet, lasagna, and various cooked dishes	<30 h
	Staphylococcus aureus, Salmonella, Shigella	9.6 CFU/g for Staphylococcus aureus, 2.0 CFU/g for *Salmonella* and 6.8 CFU/g for *Shigella*	Fresh pork	<8 h
NASBA	*Escherichia coli*	40 cells/mL	Drinking water	4 h
	Salmonella enteritidis	10^1 CFU/ reaction	Artificially contaminated fresh meats, poultry, fish, ready-to eat salads and bakery products	26 h
	Listeria monocytogenes	400 CFU/mL	Artificially contaminated cooked ham and smoked salmon slices	72 h
	Bacillus amyloliquefaciens, Bacillus cereus and *Bacillus circulans*	-	Artificially contaminated milk	Not stated
	Salmonella Enteritidis and *Salmonella typhimurium*	<10 CFU/mL	-	<90 min
LAMP	*Vibrio vulnificus*	5.4 CFU/ reaction for a virulent *V.vulnificus* in a pure culture. 2.5×10^3 CFU/g for a virulent *V. vulnificus* strain in spiked raw oyster, no enrichment. 1 CFU/g for a virulent *V. vulnificus* strain spiked in raw oyster after 6 h enrichment	Artificially contaminated raw oysters	8 h
	Salmonella spp. and *Shigella spp.*	5 CFU/10 mL	Artificially contaminated milk	<20 h
	Vibrio parahaemolyticus	10 CFU/reaction	Naturally contaminated seafood samples: fish, shrimp and mussel	16 h
	STEC O26, O45, O103, O111, O121, O145, and O157	1-20 cells/ reaction in pur culture and 10^5-10^6 CFU/25 g in produce	Artificially contaminated lettuce, spinach and sprouts	Not stated
Oligonucleotide DNA microarray	*Esherichia coli*O157: H7, *Salmonella enterica*, *Listeria monocytogenes* and *Campylobacter jejuni*	1×10^{-4} ng for each genomic DNA	Naturally contaminated fresh meat samples: chicken, beef, pork and turkey	Not stated

	Listeria monocytogenes	8 log CFU/mL	Artificially contaminated milk	Not stated
	Escherichia coli, Shigella spp., *Salmonell spp., Proteus* spp., *Campylobacter jejuni, Enterococcus faecalis, Yersinia enterocolitica, Vibrio parahaemolyticus, Vibrio fluvialis, ß-hemolytic streptococcus, Staphylococcus aureus*	10 CFU/mL of pure culture	Artificially and naturally contaminated pork, chicken, fish and milk	Not stated

Table 4: Detection of Food Borne Pathogens using conventional methods, histological and radiological techniques.

Let us look at the quick look at George's case where both a timely molecular test enables him to go home within a matter of hours.

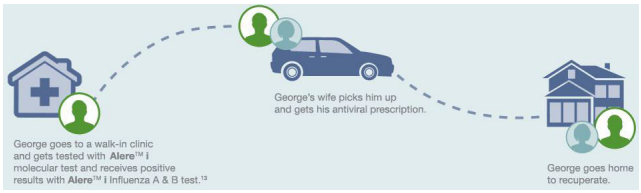

Figure 1: Fighting influenza with rapid molecular diagnostics.

Accurate Diagnosis of Infectious Diseases

Without no doubt, the use of conventional methods to study infectious diseases was a success, however, a more substantial contribution came into reality when microbial pathogenicity at the molecular level comes in [15,18]. This has created a wider understanding of clinical manifestations, diagnosis, treatment and immunoprophylaxis of infectious diseases [15].

Since traditional methods depend on morphologic and metabolic features, they often lack the ability to show the genetic diversity found among the strains [15] and this is something that Nucleic acid Amplification Techniques (NAT) excels at. NAT are the leading methods in molecular diagnosis. They are causing a big impact on the study of microbial pathogenesis and even on the infectious diseases diagnosis [15-17]. Epidemiology always cannot precede the onset of an outbreak, and before an outbreak can be recognized, the circulation of diseases agent in a population can easily be detected with molecular methods [15]. In the below study, the table compared the accuracy of the diagnosis with multiplex PCR and culture using both direct and enriched methods.

Demistifying the Cost Involved in Patient Treatment

Being hospitalized costs money, whether paid for by the government, some insurance ageny, or the individual, receiving a treatment has never been an economic treat but being hospitalized and paying for the nights-stay has always been a problem. The conventional methods although reliable enough for diagnostic, require hospitalisation before the results can be obtained. For example if a patient whose sample will be analysed using conventional methods will stay for 3 nights that will be paid at $50 per night and another $50 for the test. This patient can pay $150 dollars for molecular detection and go home the very day. To minimize the hospitalized or turnaround period, automated machines are able to send results immediatly to the information system through an interface [3,12]. In the recent past after PCR was introduced in the microbial diagnostic laboratories, most technicians thought that the costs involved cannot be sustainable. However, this is similar to the law of demand and supply in markets, with the mass production of software applications, primers and probe designs, the cost of molecular methods falls [10]. For example with nucleic acid amplifications (NAAs), there is an increase in the use of these techniques in the diagnostic laboratories [8,10,12,17]. NAA with regards to the attainment of high-quality patient care seems to be the choice since it is fairly reasonable to be performed in the microbiology laboratory [10,13]. Respiratory pathogens have been proved quicker to diagnose when using molecular methods than traditional methods [10,11].

Non-viable Culture Organisms (meaning)

Molecular methods have come as a blessing to the clinical microbiology laboratories with the slogan 'impossible is now more possible'. In the last decades, organisms that cannot be grown in culture or that are very difficult to grow for example; Hepatitis B virus (HBV), Hepatitis C virus (HCV), Human Immuno Deficiency Virus (HIV), Ebola Virus (EBV) and Cytomegalovirus (CMV) can now be efficiently detected with the use of PCR methods in a matter of hours [1]. Also due to the infectious dose, highly infectious agents that cannot be considered for culture. For example; *Francisella tularensis*, *Brucella* Spp, *Coccidioides immitis* etc. can now be identified through the molecular methods [1]. With the invention of molecular methods, a greater depth of knowlege is known about some critical organisms (fastidious) that require specialized media or culture conditions to grow [12,17]. In the recent discoveries, the information which said that only a few bacteria can be grown on artificial media has saved us from what we have been missing in specimen diagnostic. Now with culture-independent methods, both diagnostic and study of disease pathogenesis has been greatly acknowledged [12,18].

The Need to Wipe off the Tears of Developing Nations

The word 'developing' itself is challenging. Many of these nations that carries the big burden of the long historic malaria, tuberculosis and HIV and many other infectious agents cannot afford molecular methods in clinical diagnosis [2]. Usually these are due to the cost involved and the technical expertise that are unaffordable, as of today [2]. The monitoring of progression and the control of the spread of these dreadful resistant pathogens can only be accomplished with the molecular methods [2].

Our Big Hopes for the Future

The rapid detection and quantification of microbial pathogens in the clinical laboratories have exceedingly been recognised. With the introduction of PCR in clinical diagnostic laboratories, the detection and quantification of infectious microorganims, genetic disorder and cancer cells has gained the upper hand [1,3]. PCR is one-tube system that minimizes the risk of contamination. It can amplify long target up to 6.0 kb and also offer high sensitivity and can be reproducible

analysis of both DNA and RNA [18]. PCR is the most popular approach in the family of diagnostic molecular methods and it has wiped out the need for extensive specimen preparation and improved automation [3]. Multiple pathogens can be detected simultaneously with the use of multiplex platforms [3].

In short, multiplex PCR has the ability to detect number of potential pathogens simultaneously. The coming of the high-throughput sequencing (HTS) to genetics, has made a significant revolution in the diagnosis of infectious diseases. Henceforth there is a need for these methods to replace the conventional methods in the diagnostic laboratories [5].

We are hopeful that with the use of NGS the discovery of novel pathogens that might be related to acute or chronic illnesses will be achieved. Appropriate quality controls, interpretation of data and the fullfilment of regulatory requirements are considered to be crucial [13]. Direct sequencing and also microarray analysis will lead the molecular diagnosis with great hopes in both research and clinical laboratories [10]. Below are the already molecular methods but limited to, that have shown the diagnostic laboratories.

The Limitations of Molecular Methods

In the health departments and especially in the clinical diagnostic sections, a serious patient care is always needed, because a single mistake made during the diagnosis can lead to a life being lost. In this regard, despite the nobility of our faithful molecular methods in the diagnostic laboratories, we still have to cross-check its limitations.

One of the limitations is based on the species whose gene has not been included in the genome database known. Molecular methods lack the detection of this species and cannot detect if there is a newly emerging resistance mechanisms [13,16].

Second to the emergence of new genes is the unbalance nature of the microbiology laboratories between the developed and developing nations, and the non-bridging of microbiology and molecular laboratories [2].

A matter of hours can enable results or us to gain and hence treatment is halfway completed. However, a typical DNA sequencing is done over night, whereas s quick blood cultivation test can be done in a matter of hours [12].

While the accurate and advanced molecular laboratory techniques contributed to a more effective diagnosis, patient care and management in developed nations, the developing nations are still struggling with the only available ill equipped and sometimes the undertrained personnel [2,12]. It requires a concommitant techniquical know-how [12,16].

Majority of the availlable test kits are typically limited to the detection of one or a few pathogens [3,16] and this means more different kits are needed for diagnosing various agents. This is exponentially expensive. Of course, the margins between the cost of conventional and molecular diagnostic test is enough for one to go in for conventional tools especially in the clinics where numbers of sample are waiting on the bench. In addition, molecular methods are a lot more expensive than traditional methods, as seen from the healthcare system's point of view. Sending a sampe off for DNA sequencing will produce an invoice that has to be paid. However, using the traditional approach, there are no invoices (as the healthcare systemsees it), just staff doing the culturing/microscopy etc. [3]. Still on the cost involed, the fact that prolonged hospitalization also costs money, and that staff needs salary, is not factored in as a cost for tradition methods since there are no invoices for these. In short from where a healthcare system is looking, molecular methods for microbiological identification are extremely expensive (the traditional methods, after all, are for free). In some cases, molecular methods only augment cultures like; where live bacteria is needed to conduct antimicrobial susceptibility testing (AST) [3,16]. Aside from *mecA* (a gene found in bacterial cells that enable resistance of bacteria to antibiotics like penicillin and its likes) and *VanA* (a gene responsible for vancomycin-resistant), AST assays are very few at hand for specific genes [3].

Contamination of all kinds also joins in the factors of limiting the use of molecular methods in diagnostic laboratories. One of these is the carryover contamination [12]. The pre and post amplification contaminants always translated into false positivity [12,16].

Human imperfections that bring in sampling error also inhibit the assay, causing false negatives [12]. Traditional methods will still take a lead for the reasons of being tradition. Since we always have the believed that 'this is how we do it' and we will more or less prefer to be doing it like this. For example if salmonella could be culture and identified by biochemical test, going futher with confirmation with molecular tests might be less on the option [16]. However if all the laboratory technicians are well sensitived on the emergence of molecular techniques and the coniderble reduction of the cost involved, this hurdle can be overcome.

Molecular methods when compared to conventional approaches seems more expert oriented. One example is a test that involves the use of 16S rRNa gene amplification and sequencing [10]. This is the best for bacteria if the purity of sample is assured; however, it is always a hurdle if the corresponding culture is negative. Probably there are mixed infections or unusual pathogens [10]. In addition, deviant bacteria are likely to require very specialized PCR primersç; if you just use regular bacteria, you will miss them [5].

Conclusion

Clinical microbiology laboratories in the developing nations should be updated with the recent molecular techniques while revolutionized to add a more efficient diagnosis and patient care. Previous studies have revealed that severe morbidity and mortality are due to the lack of point care accurate laboratory diagnostic methods. In this response, the introduction of more sophisticated molecular methods will effectively help in the control of infectious disease epidemics and pandemics that are of public health importance. In fact, it is time for for the healthcare system to take the step over to molecular methods in, for example bacterial identification.

The accreditation of laboratories and competence of the staff in molecular diagnostic laboratories should be frequently monitored.

In addition to the need of standard protocols or standard operating procedures, reference materials and proficiency testing programmes for testing methods will give results that are more consistent among different laboratories.

With the implementation of Good Clinical Laboratory Practices (GCLP) or Health and Safety for example handwashing, reporting of compliance and with regular monitoring and surveillance of handwashing will drastically help to reduce nosocomial infections [6].

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