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# Molecular diagnostics: the changing culture of medical microbiology

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# Molecular diagnostics
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Diagnostic molecular biology is arguably the fastest growing area in current laboratory-based medicine. Growth of the so called 'omics' technologies has, over the last decade, led to a gradual migration away from the 'one test, one pathogen' paradigm, toward multiplex approaches to infectious disease diagnosis, which have led to significant improvements in clinical diagnostics and ultimately improved patient care.

### Introduction

Until recently the tools available to the clinical microbiologist were antiquated in comparison to most other biological specialties. With a basic technology essentially unchanged for decades, clinical microbiologists were limited in which organisms they could detect and as a result, it is likely that a multitude of infections were left undiagnosed. After an enduring wait, the "Molecular Revolution", which has already significantly impacted other branches of microbiology, is now being felt in clinical microbiology laboratories and promises to propel this discipline into the 21st century. At last, traditional culture-based infection diagnostic techniques are gradually being replaced by more advanced molecular detection methods, in a movement which has been hastened by rapid advances in genomics and bioinformatics. Herein, we examine the challenges, considerations and some new developments in the field of molecular clinical microbiology that are likely to affect, and be affected by, the medical scientist as part of the collective drive to improve healthcare.

Traditionally, the gold standard for diagnosis in the clinical microbiology laboratory has been culture—the organism is isolated and identified definitively followed by the generation of a patient report. It is widely accepted that the earlier an accurate microbiology report can be generated, the more significant the impact it is likely to have on patient care. However, sample culture takes a minimum of 18 h, followed by further subculture for identification and susceptibility testing. It is likely that these gold standards for diagnosis have evolved on the basis of the most sensitive and specific techniques available at the time. Now in the "omics era", the time has come for clinical scientists to reassess the components of their "diagnostic tool box" to help improve infectious disease management. Technological advances, such as molecular diagnostics, offer the possibility of rapid reporting and improvement of the impact of clinical microbiology on patient management.

### The First Wave of Molecular Diagnostic in the Clinical Microbiology Laboratory

Currently, in clinical diagnostic laboratories, molecular methods are emerging as the frontrunners in the detection of particular pathogens and thus, are beginning to replace traditional culture-based techniques. One example of where molecular-based diagnosis has already become well established is in the detection of *Chlamydia trachomatis* infection. The 1980s saw direct fluorescent antibody (DFA) tests and enzyme immunoassay replace tissue culture—the gold standard of the time—particularly in the absence of specialized and time-consuming tissue culture facilities. In the 1990s the commercial development of Polymerase Chain Reaction (PCR) and Ligase Chain Reaction (LCR) methods for chlamydia detection, although more expensive than DFA/EIA, were nonetheless shown to be comparable to tissue culture in terms of sensitivity and specificity.[1-3] More recently, Real Time PCR (RT-PCR) methods for chlamydia detection have been developed, and large throughput of samples and commercial competition have helped to drive down costs incurred by the testing laboratory, to make this a routinely-used method. The widespread use of relatively user-friendly molecular methods for chlamydia testing may have helped to pave the way, albeit slowly, for the uptake of further molecular assays as they became available.

Moreover, many of these techniques facilitate post-amplification analysis on the products. Such techniques include melt-curve analysis (MCA), in which the resolution relies on the difference in the melting temperatures ($T_m$) between amplicons. This provides different information, depending on the type of probe used. High resolution MCA may be used to detect Single Nucleotide Polymorphisms (SNPs) when using non-specific DNA binding dyes, facilitating speciation and

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source identification after broad range nucleic acid amplification.[4] The use of SNP analysis within the diagnostic microbiology laboratory is likely to have a significant contribution to the field of epidemiology. Moreover, high resolution MCA has been used in the detection of antibiotic resistant genes in bacteria such as *Hemophilus influenzae, Neisseria gonorrhoeae* and multi-drug resistant *Mycobacterium tuberculosis*.[5]

## The Cost of Molecular Diagnostics: A Worthy Price to Pay?

The fact that certain routine diagnostic tests remain traditionally culture-based is dictated largely by the cost of introducing new techniques, staff training and increased space requirements. Such factors are all too often the primary causes for stagnation when implementing novel molecular diagnostics in the clinical laboratory.[6]

The development of a CE-IVD assay, for example, incorporates a number of extensive phases including research, analytical and clinical validation, certification and ongoing post-market approval requirements. Furthermore, significant costs are incurred from developing novel technologies for an assay or through license fees for the use of patented technologies. As an example, a multiplex real-time PCR assay may incur license fees for gene targets, fluorophores, quenchers, modified polymerases, amplification technology and preparation processes such as lyophilization. Furthermore, the cost of Good Manufacturing Practice (GMP) grade raw materials, manufacturing, direct/indirect distribution and product support also bring with them important financial considerations.

However, it is likely that the economy of scale that comes from using common platforms and technicians specifically trained for molecular diagnostics facilitate an increase in workload without the corresponding increase in laboratory staff.[7] Moreover, molecular diagnostics easily accommodate the concept of consolidation, integration and automation. Also, such tests fit seamlessly into the model of an integrated laboratory which may in turn loosen the link between the microbiologist and the microbiology test, arguably to the benefit of the patient.[7]

It appears that the benefits of these assays are less likely to be found in the laboratory and more likely to be seen at the patient level by the clinician, healthcare provider or administrator.[7,8] For example, the rapid detection and characterization of an infecting pathogen may allow the clinician an opportunity to tailor antimicrobial therapy and aid the use of narrow-spectrum antibiotics as opposed to broad spectrum reagents.[9,10] In comparison to traditional microbiology culture, molecular diagnostics facilitates rapid identification of at risk individuals, allows for early detection of asymptomatic disease and facilitates the monitoring of treatment which may ultimately promote new drug design and development.[9-12] Such improvements in patient care and treatment will not only reduce the spread of antimicrobial-resistant organisms but is likely to reduce the adverse effects of broad spectrum drugs; including nosocomial antibiotic associated diarrhea (AAD) as a result of opportunistic *C. difficile* infection.[13] In effect this will have a rippling outcome on patient hospital duration and reduce the number of hospital beds occupied by patients who contract nosocomial infections.

It is apparent that new and advanced methods of molecular diagnostics are shifting the way in which we practice clinical microbiology, which in turn are likely to affect the practice of medicine in general.[8] While this "Molecular Revolution" represents an exciting departure from the norm, it remains the responsibility of both the clinician and the clinical scientist to ensure that these tests are used appropriately.[14] The large amount of data that may be obtained from patient samples using such molecular methods must be monitored carefully and strict ethical guidelines must be constructed for such tests.[15] Furthermore, the abuse of this technology has serious implications for cost as well as patient care due to the possibilities of false positive or false negative reactions.[16]

## Effecting New Strategies for Laboratory-Based Diagnosis

Overall, the recent rapid advances in molecular-based detection methods, underline the need for tripartite collaborations between clinical, research and commercial laboratories (**Fig. 1**). Collectively, we can hope to continue to design and develop ever-more sophisticated, rapid and automated assays targeting clinically significant organisms while still ideally conforming to the working day of the medical scientist. There is a fundamental need to identify those pathogens, both established and emerging, which impose the highest clinical and economic cost. Thus, the source of infection can be identified and effective infection control measures implemented swiftly enough to be of use.

A serendipitous consequence of molecular-based detection methods is that, in addition to their speed, high specificity and sensitivity, they may also facilitate the detection of previously unknown and undetected pathogens. In support of this has been the identification of *Campylobacter ureolyticus* as a novel gastrointestinal putative pathogen using a commercial multiplex-based PCR detection system in the Republic of Ireland.[17,18] Given that campylobacter is currently the most commonly-isolated bacterial pathogen worldwide, the detection of a new species in more than 20% of the total campylobacter detections among patients with acute gastroenteritis is a significant finding.

Indeed, work in our laboratory has shown that *C. ureolyticus* now surpasses *C. coli* as the second most common campylobacter species in the feces of patients presenting with gastroenteritis (at least in Southern Ireland).[19] This organism, which is incapable of growth on routine campylobacter culture, to the best of our knowledge, has never previously been reported in the faeces of patients with diarrheal illness. This is an example of the benefit that molecular diagnostics can bring to bear on the detection of fastidious organisms, which by routine culture would have been otherwise reported as false negatives. Merely detecting a bacterium or virus in a clinical sample does not of course distinguish it a true pathogen or as an innocent bystander, an innocuous component of the normal flora. However, as in the case of *C. ureolyticus*, whereby the

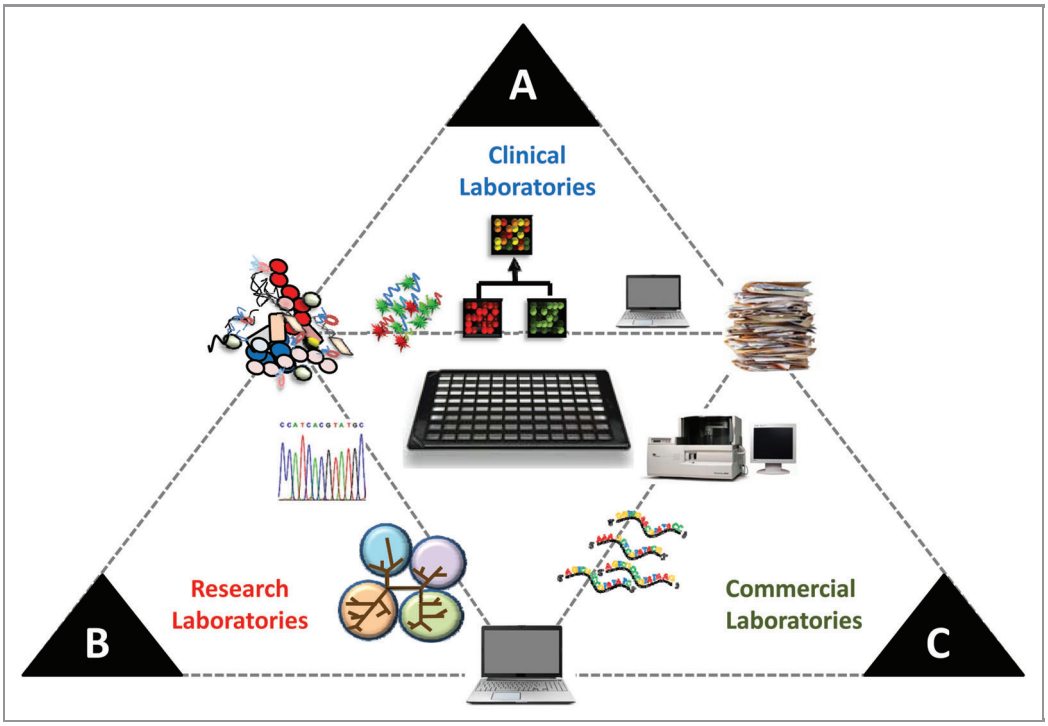


**Figure 1.** Microarray design, production and application; A Tripartite Collaboration between the Clinical, Research and Commercial Laboratories. (A) In the diagnosis and investigation of infectious disease, clinical laboratories receive patient samples from various anatomical sites with polymicrobial populations. A DNA extraction is conducted on the patient samples received and the single stranded DNA is labeled with a fluorescent tag, a single probe mixture or multiple probe mixture as seen in (A), maybe prepared using multiple fluorescent labels in a multi-color DNA and RNA hybridization. The probe sample can then be added to a commercial "disease-specific" microarray which contains a repertoire of genus and species specific oligonucleotides. The probe sample is incubated with the microarray in a hybridization station to allow molecular binding. After the probe binding reaction is complete, the microarray is washed to remove unbound material and fluorescence is observed based upon the hybridization of the labeled DNA in the patient's sample to complementary immobilized oligonucleotides on the microarray. A microarray scanning or imaging device is used to acquire the signals, followed by signal quantification yielding data. Strict inclusion and exclusion criteria are applied to the data to ensure statistical relevance. Computational programs will then apply statistical analysis on the data obtained as to whether or not clinically significant organisms were present in the patient sample, ultimately resulting in the generation of a patient report and the diagnosis or exclusion of infection. (B) In order to design beneficial diagnostic "disease-specific" microarrays, the research laboratories will communicate with the clinical laboratories, relating to sample types and clinically significant organisms which would be of interest in human health and disease. Clinical laboratories may provide research laboratories with specimen samples containing polymicrobial populations and a biological matrix which is representative of various states of health and disease. DNA extraction would be performed, followed by DNA sequencing and phylogentic analysis of the organisms detected. By assessing and comparing the pan genomes of species belonging to a particular genus and even various strains of a certain species, candidate genes can be selected that represent the core genome of the organisms of interest, along with identifying strain differences represented by accessory genes. This facilitates the design of a microarray that is capable of specific microbial species identification from a polymicrobial sample. (C) The microarrays designed by the research laboratories may then be developed further by commercial laboratories. Additional insilico analysis may refine the selected candidate genes and result in the synthesis of genus/ species/ strain specific oligonucleotide probes. Such probes can then be robotically immobilized onto glass slide. The resulting microarray will then be subjected to extensive development phases including research, analytical and clinical validation, certification and ongoing post-market approval requirements. Once the particular microarray is available commercially, a feedback system may be in place between the commercial laboratories, the clinical laboratories and the research laboratories relating to assay performance in the clinical setting and possible measures for improvement, in addition to the alteration of such assays to rapidly respond to emerging pathogens.

clinical syndrome of the patients and corresponding microbial detections are linked, a compelling case might well be made for causality. Interestingly, the majority of fecal samples (> 90%) routinely tested for bacterial enteric pathogens return negative results, begging the question—what else is being missed?

## The Dynamic Nature of Infectious Disease

Pallen and Wren[20] refer to bacterial genomes as "molecular palimpsests", whereby the variable region of the genome displays the scars of recurring rounds of gene acquisition and erosion. Recent studies have highlighted the unexpected large genetic diversity between strains of the same species, thus blurring our definition of species boundaries.[21] The merging of phylogenetics and genomics to give phylogenomics has revolutionized comparative genomics in bacteriology.[22,23]

Comparative phylogenomics presents us with a genome profile for an organism, giving it a signature genome.[23] This technology can be employed in the research laboratory to identify new "reporter genes" for bacterial identification and thus the diagnosis of infection.[24]

The phenomenon of horizontal gene transfer, pathoadaptive mutations and genome decay further emphasizes the likelihood of detecting unexpected organisms as the causative agents in human disease.[20,21,25] The core of nucleic acid testing in the clinical laboratory is PCR and although it provides rapid clinical information with a high degree of sensitivity and specificity, the adage "you only find what you're looking for" applies.[26]

As Rappuoli et al. state, "dozens of new infectious diseases are expected to emerge in the coming decade",[27] in order to rise to this challenge and combat the dynamic nature of infectious disease we will most certainly be required to develop more comprehensive identification methods in response to new microbial variables such as chromosomal shuffling. One such approach involves the use of DNA microarray technology, which enables whole-scale comparisons of bacterial genomes, thus bringing comparative phylogenomics to the forefront.[21] Furthermore, as infections can sometimes be polymicrobial, in particular many enteric infections,[19] the limit of traditional molecular multiplexing is defined by the maximal primer concentration that can be accommodated in a primer mix.

The detection of putative novel polymicrobial infection is likely, at least in the short term, to be restricted by its establishment as the definitive cause of disease. Realistically, new findings are unlikely to be accepted without first fulfilling a revised version of Koch's postulates, such as that proposed by Fredericks and Relman in 1996[28] or Molecular Koch's Postulates proposed by Falkow in 1988.[29]

## The Use of Microarray Technology in Diagnostic Microbiology

Although microarray technology was originally developed for gene expression studies, it has been adapted to the rapid assessment of the composition of complex microbial populations in array comparative genomic hybridization (aCGH).[30] It has been reported that the use of "limited microarrays" for the assessment of numerous genetic targets after either a multiplex reaction or a broad range PCR, at present is one of the most promising areas in molecular diagnostics.[8] Furthermore, DNA microarrays enable a much larger-scale comparison of bacterial genomes than conventional PCR and form a platform for comparative phylogenomics.[21]

Regardless of its technical format, this technology consists of numerous individual probe-target hybridization reactions that are tested for simultaneously. Various microarrays have been scaled down from large platforms to smaller-sized versions that address clinically important issues that may require multiple results. Regarding microarray design, the overall aim for incorporating this technology into the clinical laboratories is to design disease-specific arrays that contain approximately 30–40 hybridization sites with genus- or species-level probe sites for the common pathogens and broad-range bacterial probes for the rare pathogens. For example Luminex markets the FDA approved "Luminex RVPTM Assay", a panel of probes for detecting 12 viral agents of upper respiratory tract infections currently being used in clinical microbiology diagnostics. Additionally a DNA Microarray for the detection of bacterial pathogens in patients with chronic obstructive pulmonary disease has been described.[31] Furthermore, in situations where a specialist medical facility requires simultaneous testing for a selection of infections pertaining to its patients, it is becoming feasible to order a commercial custom-designed microarray from companies such as Bechman Coulter Genomics, Massachusetts or Arrayit Corporation, Silicon Valley, California.

In premise, by assessing and comparing the pan genomes of species belonging to a particular genus and even various strains of a certain species, we can carefully select candidate genes representing the core genome along with identifying strain differences represented by accessory genes, thus allowing for specific microbial species identification from a polymicrobial sample. Willenbrock et al.[30] for example, describes a novel pan-genome microarray encompassing 24 *E. coli* and 8 Shigella genomes with the aim of identifying emerging pathogens. Another beneficial microarray to the clinical laboratories was described by Wang et al.,[32] a long oligonucleotide DNA microarray capable of simultaneously detecting hundreds of viruses. In this particular microarray, elements were selected from highly conserved regions within viral families which still enabled individual viruses that were not explicitly represented on the microarray to be detected, thereby facilitating new unsequenced or uncharacterized virus discovery. Thus, DNA-microarrays composed of carefully selected viral sequences, coupled to a random amplification step allows for a broad reaching and unbiased detection strategy.

Moreover, the increase in genome sequencing coupled with methods such as suppression subtractive hybridization which allows for the differential comparison of bacterial genomes, allow us to move toward "pan arrays" or "whole-pan-genome tiling arrays".[33,34]

Such advances are radically transforming our current view of multiplex laboratory testing and will hopefully expand pathogen detection to include bacterial population-based analysis and host specific responses. Disease-specific microarrays, which may be a microbial detection array and/or a microbial functional gene array, will rely on using these formats after broad range PCR, multiplex PCR or a combination of such assays. Such an array would, in a single post-amplification assay, address all the most common etiological agents that may be responsible for the infection.[35,36] Thus, we must aim to identify a repertoire of candidate genes that will allow for the sensitive, selective and accurate identification of those organisms causing human disease; a challenge for both the research and medical scientist alike.

As the panels of infectious agents are continually expanding, coupled with the aid of phylogenomics to decipher the genetic clues to bacterial virulence and host specificity, a readily modifiable approach to multiparameter testing in diagnostic clinical laboratories is dawning

and along with it the advent of personalized medicine.

## Personalised Medicine: The Future of Biomedicine

The era of personalised or individualised medicine began in earnest with the publication of the draft human genome in 2001.[37-40] Genomic studies have tended to focus on the host genome, to identify host genes that are expressed after bacterial invasion or that are associated with susceptibility to infection.[20]

Taking into consideration that bacterial cells outnumber human cells by a factor of 10 to 1, one particular area that may contribute to a personalized approach in diagnostics is the investigation of the microbial species usually assigned commensal status at particular sites of the body.[41] The Human Microbiome Project (HMP) has resulted in substantial revelations regarding human and microbial interactions and the development of disease.[42] The focus of the HMP was to define a core set of microbes associated with the human body; variations in this "core microbiome" may eventually be correlated with changes in human health.[43] For example a number of studies investigating the contribution of microbial populations to human health and disease have reported a 10-fold shift in the ratio of *Firmicutes* to *Bacteroides* species in the gastrointestinal tract (GI) of obese patients in comparison to their lean counterparts.[44] Moreover, it is estimated that the GI tract is occupied by an excess of $10^{14}$ microorganisms, representing a vast ecosystem of almost 1,000 different bacterial species.[44] Additionally, the "metagenome" of the microbial species resident in the various sites of the human body is estimated to be 100-fold greater in terms of gene content than the human genome. This diverse and complex collection of genes encodes a wide array of biochemical and physiological functions that may benefit the host as well as the neighboring microbes.[45]

Acknowledging the strength and rapid response of the human immune system to infectious agents, the ability of these microbial populations to exist in synergy within the human host cannot be overlooked and we must redirect our assessment of human health toward the "human superorganism" whereby diagnostic and prognostic assessments should recognize the contribution of not only mammalian cells but also our constituent microbial cells to human health.[46]

Commercial companies such as Human Microbe Identification Microarray core (MIM) (based at The Forsyth Institute, Cambridge, Massachusetts, USA) promise to allow for the rapid determination of bacterial profiles, to the species level, from clinical samples of the human oral cavity.[47] Although currently only permitted for use in research laboratories, this Human Oral Microbe Identification Microarray (HOMIM) enables the simultaneous detection of approximately 300 of the most prevalent bacterial species in the oral cavity, thus permitting the comparison of bacterial associations in health vs. disease.[47-50] At present, their research has linked variations in oral microbial populations not only with periodontal health but also with inflammatory bowel disease and pancreatic diseases including pancreatic cancer.[49,50] Furthermore, Forsyth state on their website (mim.forsyth.org) that they are currently developing microarrays to detect the combinations of bacterial species in the human gastrointestinal tract, which in conjunction with the findings of the HMP are likely to provide valuable insights into the "human superorganism".

With an alarming annual average of more than 13 million deaths worldwide from infectious diseases,[51] partially due to an increase in antibiotic resistance in infectious organisms, new approaches, such as pharmacogenomics, tailoring host-pathogen-drug interactions are emerging to circumvent such resistance. Pharmacogenomics, a promising area in personalized medicine introduces the concept of molecular markers (biomarkers) to assess drug efficacy, safety and disease risk.[52] It promises to predict the individual outcome of specific therapies in addition to broad pharmacological strategies while considering both host and pathogen to determine the "right drug" for the "right patient" at the "right time". For example individualized antibiotic treatment has already been initiated by considering drug-metabolism enzyme variants.[51] Moreover, recent studies are extending such treatment by focusing on SNP's, haplotyping, particular gene copy numbers and the individual response of the host's immune system.[52-54] Variations in the human multidrug-resistance gene-1 (MDR1) for example, such as a C to T SNP at position 3435, can predict therapy response to highly active antiretroviral therapy (HAART) in HIV-1 patients.[55] Furthermore, although abacavir is an effective treatment for HIV, fatal hypersensitivity reactions have been linked with particular HLA alleles.[56]

A significant goal for personalized/individualized medicine is to permit a shift in emphasis from disease treatment to prevention.[57] However, despite the aforementioned advances, it is fair to say that personalized medicine is still in its infancy and will require extensive validation and assessment of variables before it is anywhere near reaching its full potential in routine diagnostics.

## Conclusion

With a time-to-result of hours rather than days, molecular detection promises speed in addition to improved specificity and sensitivity. However, given that molecular biology targets DNA rather than live cells, the approach can sometimes be too sensitive—making no distinction between DNA from live or dead cells. Ultimately, the effective use of molecular diagnostic methods will rely on the same criteria as for traditional culture based methods—maintaining an affordable level of sensitivity and specificity, and seeking not to exclude the less common pathogens, while selecting for those deemed to be the most significant in human infectious disease.

Diagnostic molecular biology is arguably the fastest growing area in current laboratory-based medicine, and has the potential to change the course of clinical medicine dramatically over the next decade. Although personalized medicine may not be ready for immediate implementation in the clinical laboratories as yet, it is likely that its progression will be coupled with the advances seen in molecular methods, thus allowing us to define the scope and nature of human biological variation and permit us to target medical

treatment to those who will most likely benefit. In this era of "omics" technologies, we may soon be able to move away from the "one test, one pathogen" paradigm toward multiplex approaches to infectious disease diagnosis and in doing so, revolutionize clinical diagnostics and ultimately improve patient care.


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