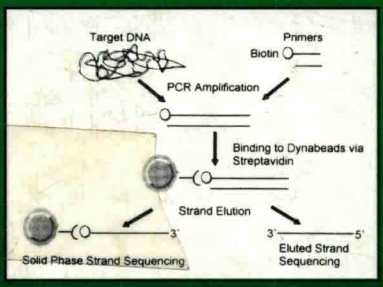
METHODS IN MOLECULAR MEDICINE™

Molecular Bacteriology

Protocols and Clinical Applications

分子细菌学方法与临床应用

Edited by
Neil Woodford
and Alan P. Johnson



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by Neil Woodford et al.

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Molecular Bacteriology: *Protocols and Clinical Applications*

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Preface

The enormous advances in molecular biology that have been witnessed in recent years have had major impacts on many areas of the biological sciences. Not least of these has been in the field of clinical bacteriology and infectious disease. *Molecular Bacteriology: Protocols and Clinical Applications* aims to provide the reader with an insight into the role that molecular methodology has to play in modern medical bacteriology.

The introductory chapter of *Molecular Bacteriology: Protocols and Clinical Applications* offers a personal overview by a Consultant Medical Microbiologist of the impact and future potential offered by molecular methods. The next six chapters comprise detailed protocols for a range of such methods. We believe that the use of these protocols should allow the reader to establish the various methods described in his or her own laboratory. In selecting the methods to be included in this section, we have concentrated on those that, arguably, have greatest current relevance to reference clinical bacteriology laboratories; we have deliberately chosen not to give detailed protocols for certain methods, such as multilocus enzyme electrophoresis that, in our opinion, remain the preserve of specialist laboratories and that are not currently suited for general use. We feel that the methods included in this section will find increasing use in diagnostic laboratories and that it is important that the concepts, advantages, and limitations of each are thoroughly understood by a wide range of workers in the field. To assist in this, the subsequent chapters in the volume describe the application of these and other methods to the investigation of a variety of bacterial pathogens, diseases, and antimicrobial resistances. Our aim is that by cross-referring between chapters, the reader should become conversant with both the practical and theoretical aspects of the topics covered.

We believe that *Molecular Bacteriology: Protocols and Clinical Applications* will provide a valuable source of information for workers in both clinical and academic settings. In particular, we feel the Notes sections included at the ends of most of the chapters should prove to be of particular interest as they often include "tricks of the trade," that the various contributors have learned through personal experience.

Neil Woodford
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Contents

1

Impact of Molecular Methods on Clinical Bacteriology

Robert C. George

1. Introduction

The impact of molecular (nucleic acid-based) methods on the basic science of medical microbiology is undeniable. Indeed, microbiologists have been at the forefront of the molecular biology revolution that has had such a dramatic effect on our understanding of biological science. Although the foregoing is indisputable, have these techniques yet found an appropriate, cost-effective, and quality-assured place in the clinical bacteriology laboratory? Are patients and the infections from which they may be suffering managed more effectively and efficiently through the application of molecular methods? This introduction seeks to explore these issues from the perspective of a clinical bacteriologist. Detailed theoretical and practical guidance on the application of these techniques to the diagnosis, management, and epidemiology of a wide range of infections is provided in the succeeding chapters.

In very broad terms, the functions of a clinical bacteriology laboratory are twofold: first, the examination of biological samples (and the organisms isolated from, or detected in them), to determine the etiological diagnosis, specific treatment, and control of bacterial infections; and second, the formulation of specific and general advice, guidance, and policy for the management, control, and prevention of bacterial infections in individuals and communities. In considering the impact of molecular methods on clinical bacteriology, this chapter will concentrate more on the former than the latter functions of the diagnostic laboratory. However, new insights provided by these novel technologies—in particular, rapid and simple methods for microbial "fingerprinting" and/or detection of particular antimicrobial resistance or virulence genes for epidemiologic purposes—may be expected to have a significant impact on infection control policies and their implementation.

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As noted above, the impact of molecular methods on our understanding of the basic science of clinical bacteriology has been significant and will become increasingly so. However, at present many of these techniques are of greater relevance and use to the reference or other specialist laboratory than to the clinical laboratory. Some significant exceptions to this general statement are the following: laboratories serving tertiary referral hospitals that with their particular patient populations and clinical specialities, can make cost-effective use of molecular methods in diagnosis, therapy, and epidemiology, and, of course, clinical virology laboratories. The latter have embraced rapid molecular diagnostic technologies far more speedily and comprehensively than their bacteriology counterparts. There are several reasons for this; in particular, the specialized, skill-dependent, and retrospective nature of many conventional virological methods and the increasing number of viral infections amenable to specific antiviral or immunomodulation therapy. As a consequence, rapid, sensitive, and specific viral diagnostic and therapeutic monitoring methods are required for the effective use of these therapies. It is noteworthy that commercial suppliers of molecular diagnostics have targeted this market far more aggressively and successfully than clinical bacteriology.

2. Areas of Potential Impact on Clinical Bacteriology

The molecular methods actually or potentially applicable in clinical bacteriology laboratories include the polymerase chain reaction (PCR) or other DNA amplification techniques and/or gene probing methods for the identification of bacteria and specific virulence or antimicrobial resistance genes (either in cultures isolated by conventional methods or directly in clinical material), and genomic analysis by one or more of a range of techniques for bacterial "fingerprinting" and typing for epidemiologic purposes.

The uptake and impact of molecular methods will, in part, be dictated by the clinical necessity or epidemiologic requirement for a truly rapid or otherwise unachievable result and the implication of that result for the individual patient and health-care staff. For certain infections, particularly those acquired in hospitals or those of wide and general public health significance, a positive result may have widespread ramifications. Increased speed and sensitivity in achieving that result—whether it is an etiological diagnosis, the detection of a specific virulence determinant or antimicrobial resistance gene(s), or the definition of the degree of relatedness of isolates from episodes of presumed hospital or community crossinfection—allows the implementation of appropriate therapeutic and control measures more rapidly than might otherwise be possible. It is in these areas of clinical bacteriology that molecular methods may be expected to have the greatest impact on medical practice.

2.1. Impact on Laboratory Methods for Diagnosis and Pathogen Identification

For the vast majority of common bacteriologic investigations undertaken in clinical laboratories on samples from immunocompetent individuals, biological amplification by overnight culture using simple agar or broth media is the method of choice and is likely to remain so. Notable exceptions include slow-growing or difficult-to-culture organisms (e.g., mycobacteria and chlamydiae) and infections in the immunocompromised, for which diagnostic accuracy and speed are essential and can be lifesaving.

The greatest scope for widespread application of molecular methods in routine bacteriology is in the further examination and identification of agar-grown pure cultures. The last 20 years have seen an ever-increasing acceptance and use in the clinical bacteriology laboratory of a wide range of commercially produced test "kits" for these purpose. Such kits have simplified and standardized phenotypic testing. It is therefore likely that conveniently packaged, competitively priced, and quality-assured DNA-based identification and other test systems will find a ready market.

2.1.1. Identification and Characterization of Isolated Bacteria

In essence, these will be new ways of doing old tests on agar-grown pure cultures. Speciation by DNA amplification methods and/or gene probing may replace biochemical or other phenotypic identification procedures. For certain organisms, in addition to speciation, it is also necessary to determine their pathogenic potential by demonstrating the presence or absence of certain factors (e.g., diphtheria toxin in isolates of *Corynebacterium diphtheriae*). A positive result will substantiate the diagnosis and may define the course of clinical and epidemiologic management. In such circumstances, speed of detection may be very important and molecular methods have much to offer over conventional phenotypic tests.

As such, tests will be undertaken with large amounts of target DNA obtained from bacterial colonies. Crosscontamination of reagents and equipment are of perhaps slightly less concern than for the application of molecular diagnostic methods, such as PCR, directly to clinical samples where target DNA may be present in vanishingly small amounts. Laboratory managers who have to ensure quality assurance and control of all aspects of the work undertaken will almost certainly wish to use commercial kits with built-in controls and validation steps. Determining factors in any widespread successful application of these methods will be the total costs of reagents, dedicated equipment, and facilities, as well as the training, skill base, and number of staff required to operate them. Clearly, there is ample scope for cost-beneficial automation of such test systems

with colorimetric, fluorimetric, or other machine-readable endpoints. For the majority of potential applications in this general sphere of activity, speed of testing is perhaps slightly less relevant, because many conventional phenotypic test kits for bacterial identification and characterization already give same-day results.

2.1.2. Detection of Pathogens in Clinical Samples

Molecular methods offer the promise of rapid and direct detection of bacterial pathogens in clinical material and, for a few infections, this promise has begun to be realized. Researchers, both in the commercial and public sectors, have concentrated their attention on slow-growing or difficult-to-culture organisms, such as *Mycobacterium tuberculosis* and *Chlamydia trachomatis*. Semiautomated commercial systems utilizing DNA amplification are available for the diagnosis of these latter infections and are increasingly utilized. The advantages, particularly in speed of diagnosis over conventional culture methods for slow-growing and difficult-to-culture organisms, are obvious and offer new opportunities for early clinical and epidemiologic interventions in the management of both individual patients and communities. However, rapid microbial evolution in response to ecological pressures, such as antibiotic use and advances in medical care, is occurring constantly in organisms of relevance to clinical bacteriology. Therefore, it is difficult to envisage whether a nonculture method will ever provide the same actual or potential information as a bacterial isolate.

Any relevant literature search on this subject will reveal numerous publications. However, a close analysis reveals that many of these published studies are technical evaluations of the potential of these methods, using artificially "spiked" samples or retrospective analyses rather than real-time, clinical outcome-based studies. As a consequence, and with certain specific exceptions, considerably more work is required before such techniques are likely to replace conventional methods. As always in consideration of any new diagnostic method, issues of sensitivity and specificity are paramount and, if nonculture molecular methods are to replace rather than complement standard culture techniques, they will need to be at least as sensitive and specific. Sensitivity, which can usually be improved through various technical manipulations of the sample(s) and test conditions, is ultimately unlikely to be a limiting factor. Specificity is rather more problematic and a recently published example of misdiagnosis by PCR of cerebral nocardia infection in a renal transplant patient with suspected cerebral toxoplasmosis is illustrative ***(1)***. Primers for the P30 gene of *Toxoplasma gondii* as target gave positive results with material from a cerebral abscess, apparently confirming the clinical diagnosis. However, conventional culture of the abscess material revealed *Nocardia asteroides* and subsequent PCR with the *T. gondii* P30 gene primers, and DNA from the

N. asteroides yielded an amplicon of the expected size; an example of unrelated, but clinically significant, crossreactivity leading to misdiagnosis. A subsequent publication *(2)* by the originators of the *T. gondii* P30 gene PCR pointed out that the primer sequences for this gene were unique to *T. gondii* according to published data at the time of their original publication in 1990. Furthermore, crossreactivity studies also showed amplicons of the expected size with *Plasmodium* spp. and *M. tuberculosis*, in addition to *N. asteroides*. It is self-evident that primers can only be selected for specificity according to what is published at the time of primer selection and that this body of knowledge is expanding at an exponential rate. Therefore, in addition to crossreactivity and specificity studies with species related to the target pathogen(s), the originators of new molecular diagnostic tests must consider organisms likely to be found in the same or similar anatomical sites and clinical conditions.

An important and expanding application of molecular methods is in the diagnosis of partially treated infections in which conventional culture has been compromised by prior antimicrobial therapy (e.g., meningococcal meningitis treated with penicillin in advance of hospital admission and diagnostic sample collection). Confirmation of a clinical diagnosis of meningococcal infection by, for example, PCR is desirable both for the individual patient and also in view of the public health control measures that are necessary to prevent further cases. Similarly, DNA amplification techniques offer considerable promise in the diagnosis of pneumococcal infections, for which conventional culture is often negative in patients presenting to hospitals after partial treatment in the community. In both of these examples, molecular methods offer not just a rapid diagnosis, but also the potential to make a specific etiological diagnosis that would not otherwise be possible. As new vaccines are developed and used widely it will become increasingly important to diagnose meningococcal and pneumococcal infections accurately and specifically, in order to define and characterize anticipated changes in their epidemiology.

Another important area for consideration in the design and application of molecular methods to primary diagnostic specimens is the type of sample being examined. The detection of single pathogens in normally sterile site specimens of relatively standard composition (e.g., pneumococci or meningococci in blood or cerebrospinal fluid; CSF) presents fewer technical and specificity problems than searching for evidence of one or more of several potential pathogens in complex and variable nonsterile site samples (e.g., *Legionella pneumophila* or *Mycoplasma pneumoniae* in sputum). All diagnostic DNA amplification methods applied directly to clinical samples should include internal controls for each sample to ensure that inhibition of amplification, which could result in false-negative results or significant reduction in sensitivity, is detected. In addition, and in contrast to the application of molecular