Molecular Diagnostics of Medically Important Bacterial Infections

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Abstract

Infectious diseases are common diseases all over the world. A recent World Health Organization report indicated that infectious diseases are now the world's biggest killer of children and young adults. Infectious diseases in non-industrialized countries caused 45% in all and 63% of death in early childhood. is by. In developed countries, the emergence of new, rare or already-forgotten infectious diseases, such as HIV/AIDS, Lyme disease and tuberculosis, has stimulated public interest and inspired commitments to surveillance and control. Recently, it is reported that infectious diseases are responsible for more than 17 million deaths worldwide each year, most of which are associated with bacterial infections. Hence, the control of infectious diseases control is still an important task in the world. The ability to control such bacterial infections is largely dependent on the ability to detect these aetiological agents in the clinical microbiology laboratory.

Diagnostic medical bacteriology consists of two main components namely identification and typing. Molecular biology has the potential to revolutionise the way in which diagnostic tests are delivered in order to optimise care of the infected patient, whether they occur in hospital or in the community. Since the discovery of PCR in the late 1980s, there has been an enormous amount of research performed which has enabled the introduction of molecular tests to several areas of routine clinical microbiology. Molecular biology techniques continue to evolve rapidly, so it has been problematic for many laboratories to decide upon which test to introduce before that technology becomes outdated. However the vast majority of diagnostic clinical bacteriology laboratories do not currently employ any form of molecular diagnostics but the use such technology is becoming more widespread in both specialized regional laboratories as well as in national reference laboratories. Presently molecular biology offers a wide repertoire of techniques and permutations of these analytical tools, hence this article wishes to explore the application of these in the diagnostic laboratory setting.

Introduction

The last ten years of the twentieth century allowed for an exponential increase in the knowledge of techniques in molecular biology, following the cellular and protein era of the 1970s and 1980s. This explosion of technologies from the primary discipline of molecular biology has had major consequences and has allowed for significant developments in many areas of the life sciences, including bacteriology. Molecular bacteriologists are now beginning to adopt general molecular biology techniques to support their particular area of interest. This chapter aims to examine the current situation with regard to the application of molecular biology techniques in the area of medical bacteriology, and is primarily concerned with the molecular identification of causal agents of bacterial infections. The chapter also aims at giving a broad overview of the application of current technology so that the reader has a more comprehensive overview of the diversity of techniques that are available, either as research tools or which may be used in a routine setting. The requirements of adopting molecular diagnostics in terms of management, cost, labour and space will be discussed. This chapter is a presentation of the development and the current knowledge, literature, and recommendation about the laboratory diagnosis of infectious diseases. The focus of this review is on medically important bacterial infections. The overall aim of this chapter is to provide an appreciation of the role molecular diagnostics has in routine clinical microbiology and how best these techniques can be integrated in order to enhance the healthcare system.

Historical perspective

In 1676, Anton van Leeuwenhoek, a Dutch cloth merchant and amateur lens grinder, first observed living microorganisms using his simple microscope, which he called "animalcules". He examined "animalcules" in the environment, including pond water, sick people and even his own mouth and found that these "animalcules" existed everywhere. He described and recorded all the major kinds of microorganisms: protozoa, algae, yeast, fungi, and bacteria in spherical, rod, and spiral forms. His discoveries opened up a new world namely the microbial world and this was the first milestone in the history of diagnostic microbiology.

Although the suggestion that disease was caused by invisible living creatures was made by the Roman physician Girolamo Fracastoro in 1546, people did not clearly recognize the role of microorganisms in diseases, until 1876, after 200 years that Leeuwenhoek found his little "animalcules", the German physician, Robert Koch established his famous "Koch's postulates" according to the relationship between *Bacillus anthracis* and anthrax. Koch's postulates include:

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a. In order to prove that a certain microbe is the cause of a certain disease, that same microbe must be found present in all cases of the disease.
b. This microbe must then be completely separated from the diseased body and grown outside that body in a pure culture.
c. This pure culture must be capable of gibing the disease to healthy animals by inoculating them with it.
d. The same microbe should then be obtained from the animals so inoculated, and then grown again in a pure culture outside the body.

Koch was a great pioneer in medical microbiology and his postulates are still considered fundamental to bacteriology even today. Communicable diseases can occur in populations and cause epidemics or even pandemic problems. Some outbreak diseases are so serious that they can cause hundreds, thousands, even millions of deaths, in epidemic or pandemic proportions. For example, bubonic plague, caused by *Yersinia pestis*, originally spread from Asia and was carried by rat-fleas via the ports of the Black Sea to Europe, which caused 42 million deaths, 25 million in Europe, in just less than five years between 1347 and 1352, reducing the population of Europe to 50 million. The scourge of tuberculosis was about to appear. Bunyon's epithet, "*The Captain of the Men of Death*", tells of the overwhelming fear that the disease was associated with, even until today. Until the present era, it was recognized and feared as one of the most common and most serious hazards of life, one from which escape was almost impossible. At the same time, the well-known Renaissance physician, Jean François Fernel was performing some early and important studies on the circulatory system. In Fernel's book *Medicini*, he describes descriptions of several pathological conditions, including what is now believed to be some of the earliest published accounts of endocarditis.

Comparing conventional detection methods of which have been developing over a century, molecular detection methods are relatively very young, with a history of approximately no more than 20 years old. Although deoxyribonucleic acid, or DNA, was discovered in the late 1860s, it was not used until the restriction enzyme and the recombinant DNA techniques were discovered in the 1970s. During this time, many scientists worked diligently to find the profound mystery of DNA. To trace the development of molecular detection methods, we should remember some pioneers and their discoveries.

In 1869, Johann Friedrich Miescher, a Swiss physician, discovered a weakly acidic substance of unknown function in the nuclei of human white blood cells, this substance, which was later named, deoxyribonucleic acid, or DNA. The substance was largely ignored for nearly a century because it seemed too simple to serve any significant purpose. This view changed dramatically in 1949, when Erwin Chargaff, a biochemist, reported that DNA composition was species specific; that is, that the amount of DNA and its nitrogenous bases varied from one species to another. In addition, Chargaff founds that the amount of adenine equalled the amount of thymine, and the amount of guanine equalled the amount of cytosine in DNA, from every species. During this time scientists discovered that chromosomes, which were known to carry hereditary information, consisted of DNA and proteins. In 1928, Franklin Griffith, a British medical officer, discovered that genetic information could be transferred from heat-killed bacteria cells to viable organisms. This phenomenon, called transformation, provided the first evidence that the genetic material is a heat-stable chemical. In 1944, Oswald Avery, a Canadian physician and bacteriologist, and his colleagues McCarty and Colin MacLeod, identified Griffith's transforming agent as DNA. Experiments conducted throughout the 1940s showed that DNA actually seemed to be the genetic material. However, it was still not known what the structure of DNA was, and how such a molecule could contain all the information needed to produce a human being or other living organisms, until 1953, when James Watson and Francis Crick discovered the molecular structure of DNA. After building successive scale models of possible DNA structures, they deduced that it must take the twisted-ladder shape of a double helix. The sides of the ladder consist of a "backbone" of sugar and phosphate molecules. The nitrogen-rich bases, A, T, G and C, form the "rungs" of the ladder on the inside of the helix. The pair discovered that base A would only pair with T, while G would only pair with C. They were awarded the Nobel Prize in Physiology or Medicine in 1962 for their discovery, shared with Maurice Wilkins, whose work with Rosalind Franklin on X-ray crystallography had provided further crucial evidence. In 1961, François Jacob and Jacques Monod develop a theory of genetic regulatory mechanisms, showing how, on a molecular level, certain genes are activated and suppressed and they were awarded the Nobel Prize in Physiology or Medicine in 1962 for their contribution. In 1961 Marshall Nirenberg, a young biochemist at the National Institute of Arthritic and Metabolic Diseases, discovered the first "triplet"—a sequence of three bases of DNA that codes for one of the twenty amino acids that serve as the building blocks of proteins. Subsequently, within five years, the entire genetic code was deciphered. At end of 1960s, almost all about the DNA structures and functions were understood in theory, but people still could not get any gene as they wanted or change any gene as they needed until 1970s when some in important enzymes were discovered. In 1970, Hamilton Smith, an American microbiologist, isolated the first restriction enzyme, an enzyme that cuts DNA at a very specific nucleotide sequence. Over the next few years, several more restriction enzymes were isolated. He shared the Nobel Prize in Physiology or Medicine with Werner Arber and Daniel Nathans in 1978 for his discovery. In 1972, Paul Berg assembled the first DNA molecules that combined genes from different organisms. Results of his experiments represented crucial steps in the subsequent development of recombinant genetic engineering. In 1980, Paul Berg shared the Nobel Prize in Chemistry with Walter Gilbert and Frederick Sanger, for "his fundamental studies of the biochemistry of nucleic acids, with particular regard to recombinant DNA." In 1973, Stanley Cohen and Herbert Boyer combined their efforts to create the

construction of functional organisms that combined and replicated genetic information from different species. Their experiments dramatically demonstrated the potential impact of DNA recombinant engineering on medicine and pharmacology, industry and agriculture. Walter Gilbert (with graduate student Allan M. Maxam) and Frederick Sanger, in 1977, working separately in the United States and England, developed new techniques for rapid DNA sequencing. The methods devised by Sanger and Gilbert made it possible to read the nucleotide sequence for entire genes, which run from 1,000 to 30,000 bases long. For discovering these techniques Gilbert and Sanger received the Albert Lasker Medical Research Award in 1979, and shared the Nobel Prize in Chemistry in 1980 (Genome News Network, http://www.genomenewsnetwork.org). In the 1970s, nucleic acid hybridization methods were mostly used methods and DNA probes were the powerful tools in molecular biology, microbiology, virology, genetics, and forensics etc. Although hybridization methods are highly specific for detecting targets, they are limited by their sensitivity. To get more sensitive and specific methods, Kary Mullis conceived and helped develop polymerase chain reaction (PCR), a technology for rapidly multiplying fragments of DNA in 1983 and he was awarded the Nobel Prize in Chemistry in 1993 for this achievement. In 1985, Saiki and his colleagues first used the new method to detect patient's β-globin gene for diagnosis of sickle anaemia. In 1987, Kwok and colleagues identified human immunodeficiency virus (HIV) by using PCR method and this was the first report the application of PCR in clinical diagnosis infectious disease.

Over the past 20 years, molecular techniques have been developed very broadly and fast. The nucleic acid amplification technology has been opened a new century for microbial detection and identification. At present, molecular detection methods, especially PCR-based methods, have become more and more important detection methods in the clinical diagnosis laboratory setting.

Applications of molecular diagnostic identification

Molecular identification should be considered in three scenarios, namely (a) for the identification of an organism already isolated in pure culture, (b) for the rapid identification of an organism in a diagnostic setting from clinical specimens or (c) for the identification of an organism from non-culturable specimens, e.g. culture-negative endocarditis.

Difficult to identify organisms

Most modern clinical microbiology diagnostic laboratories rely on a combination of colonial morphology, physiology and biochemical/serological markers, for their successful identification either to the genus level or more frequently to the species level. It is important that organisms are correctly identified for a number of reasons, including the correct epidemiological reporting of causal agents in a given disease state, as well as for infection control purposes. Sometimes it is argued that physicians may simply accept the Gram result and corresponding antibiogram in order to determine the optimum management plan of an infected patient. This lack of identification ability requires caution,

for the two reasons stated above. Consequently, there is a need to reliably identify organisms of clinical significance in a cost effective and timely manner. Most laboratories today rely on identification through biochemical profiling with the API-identification schema, as well as with the BBL-Crystal system, although there are several additional phenotypic systems, which are commercially available. Employment of one or a combination of such phenotypic schemes offers the diagnostic microbiologist a result in virtually all clinical situations, however, there is a small number of organisms where such methods are unable to give a reliable identification, e.g., the non-fermenting Gram-negative rods.

Rapid identification from clinical specimens

Traditional culture may take several days to allow sufficient growth of an organism so that a positive identification may be subsequently made. Often in clinical microbiology, such allowances in time result in patients being managed empirically, until the culture result is known, which may allow for the sub-optimal management of some patients. However, there is a role for molecular identification techniques from clinical specimens where a culture would give you a comparable result, but several days later. Hence, there is a role for such techniques in outbreak settings, as well as in today's world of potential bioterrorist attack.

Identification from culture-negative specimens

Since its origins in the late 19th century, bacteriology has largely been based on the ability to culture organisms of interest usually under *in vitro* laboratory conditions. The forefathers of bacteriology including Pasteur and Koch were ardent exponents of bacteriological culture and the affinity between the bacteriologist and laboratory culture has remained strong for the past 100 years. Indeed, it may be argued that the historical success of bacteriology has been a direct result of bacteriological culture, as well as its widespread adoption throughout the world. Today the ability to culture bacteria *in vitro* remains the cornerstone of this discipline. However, there are several situations where molecular approaches should be considered where conventional culture fails to identify the causal organism due to one or more of the following reasons including (i) prior antibiotic therapy, e.g. treatment of acute meningitis with i.v. benzylpenicillin, (ii) where the organism is fastidious in nature, such as the HACEK group of organisms in the case of endocarditis, (iii) where the organism is slow growing, e.g. *Mycobacterium* spp., (iv) where specialized cell culture techniques are required, e.g. *Chlamydia* spp. and *Coxiella burnetti*.

Molecular methods may be included in the diagnostic laboratory's diagnostic algorithm, thus enabling laboratories to make a rapid and reliable identification. Several molecular approaches may be adopted to help with the identification.

Description of molecular techniques employed in medical microbiology

Nucleic acid hybridization is based on the ability of two single nucleic acid strands that have complementary base sequences to specifically bond with each other and form

a double-stranded molecule, or duplex or hybrid. The single-stranded molecules can be RNA or DNA, and the resultant hybrids formed can be DNA-DNA, RNA-RNA, or DNA-RNA. Hybridization assays require that one nucleic acid strand (the probe) originates from an organism of known identity and the other strand (the target) originates from an unknown organism to be detected or identified. The probes are capable of identifying organisms at, above, and below the species level. Hybridization reactions can be done using either a solution format or solid support format. The solid support formats include: filter hybidizations, sandwich hybridizations, and in situ hybridizations. Nucleic acid hybridization methods were developed in the 1970s, and they are still used in microbial detection and identification today. They are also the important detection tools in real-time PCR such as TaqMan and LightCycler platforms.

Polymerase chain reaction (PCR)

Principle of PCR
Polymerase Chain Reaction (PCR) is an enzyme-driven, primer-mediated, temperature-dependent process for replicating a specific DNA sequence *in vitro*. The principle of PCR is based on the repetitive cycling of three simple reactions, the conditions of which vary only in the temperature of incubation. The three simple reactions include:

1. Denaturing: When the temperature is raised to around 95°C, template DNA double strand is separated to two single strands.
2. Annealing: When the temperature reduces to approx. 55 °C, two specific oligonucleotide primers bind to the DNA template complementarily.
3. Extension: When the temperature rises to 72°C, DNA polymerase extends the primers at the 3' terminus of each primer and synthesizes the complementary strands along 5'to 3' terminus of each template DNA using desoxynucleotides containing in media. After extension, two single template DNA strands and two synthesized complementary DNA strands combine together forming two new double strand DNA copies. After extension, the reaction will repeat above steps. Each copy of DNA may then serve as another template for further amplification. PCR products will be doubled in each cycle. After n cycles (approx. 30), the final PCR products will have 2^n copies of template DNA in theory and it just needs few hours.

Specific PCR
Specific PCR is the simplest PCR approach of which is designed for detecting specific target microbes. In specific PCR, primers are designed complimentary to a known DNA target and specific for the microbe being assayed. This is a key point for specific PCR so that the primers should be so-designed so that they are strictly specific for the targeted microorganisms. As the result is specific for the detection of target microbes, this method can be used as a direct detection and identification method. This is the most widely used method in the diagnosis of infectious diseases. Many organisms, such as *Mycobacterium tuberculosis*, pneumococci, meningococci and *Burkholderia cenocepacia*, can be identified by specific PCR directly.

Multiplex PCR
In multiplex PCR, two or more primer pairs are included in one reaction tube and two or more DNA templates are targeted simultaneously. This is a relatively simple molecular way to detect few different bacteria in one PCR reaction. In multiplex PCR, the primer pairs should be specific to the target gene and the PCR products should be in different sizes.

Nested/semi-nested PCR
In this approach, genomic template DNA is amplified with two sets of primers. The first PCR set produces a larger PCR product than that in second PCR set. The second PCR set uses the first PCR product as template DNA to amplify an internal region of DNA during the second (nested/semi-nested) amplification stage. The primers in the second PCR set can be different to the first set (nested) or one of the primers can be the same as the first set (semi-nested). This method can be used to increase the sensitivity of detection or to identify the first set PCR products when the primers in the second PCR reaction are species-specific.

Broad range PCR
Broad range PCR is a very useful approach for detecting microbes universally. The primers in broad range PCR are selected from the conserved regions of a particular gene that is shared by a given taxonomic group. This crucial important for broad range PCR to select real broad range primes. As the 16S rRNA gene is found in all bacteria and contains certain conserved regions of sequence, it has been mostly used as the broad range PCR target gene for detecting bacteria. The 23S rRNA is similar with the 16S rRNA gene and it may also be used to a lesser extent, as the broad range PCR target gene. Given that, there is relatively limited information regarding the 23S rRNA gene, this gene locus gene is not so popular, in comparison with the 16S rRNA gene. Another region of the rRNA machinery that is used in broad range PCR is the inter-spacer (ITS) region between 16S and 23S rRNA genes. In this broad range PCR approach, the forward primer is from 16S rRNA gene, and the rewords primer is form 23S rRNA gene. An example of the arrangement of the rRNA operons is illustrated with the obligate Gram +ve intracellular pathogen, *Tropheryma whipplei*.

1. Nested PCR: After broad range DNA amplification, species-specific primers are used in tandem with a second set of DNA amplification primers. The result can be detected by standard gel electrophoresis.
2. DNA probe hybridization: The broad range PCR products can be identified by using species-specific DNA probe hybridization. The probe and the PCR products are incubated together in a single test tube, and the binding of probe to the target is measured.
3   DNA enzyme immunoassay (DEIA): In this method,

an anti-dsDNA antibody, particularly recognizes the hybridization product, resulting from the reaction between target DNA and a DNA probe. The final product is revealed by means of a colorimetric reaction. The DEIA increases the sensitivity of a previous PCR by including enzymatic reactions. The hybridization between specific probe and PCR-amplified target DNA, as well as the formation of target DNA/probe hybrids and anti-dsDNA antibody complex, also enhances the specificity.

4. Single-strand conformation polymorphism (PCR-SSCP): SSCP generally is used as a microbe typing and mutation detection method. It can also be used for the purposes of microbe identification. After PCR products are denatured to two single-stranded DNAs, the physical conformational changes in single-stranded DNA are based on the physiochemical properties of the nucleotide sequence. Conventionally, the variations in the physical conformation are detected with non-denaturing polyacrylamide gel electrophoresis and stained with silver. Also, the result can be detected by using fluorescence-labelled primers and analysed on an automated DNA sequencer,

5. Restriction endonuclease digestions (PCR-RFLP): After restriction endonuclease digestions, the amplified DNA fragments are cut to different small fragments according to their DNA sequences. The resulting fragments can be separated by gel electrophoresis, and/or then transferred to a nylon membrane. RFLP usually is used as a microbe typing and epidemiological investigative method.

Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR is the technique of synthesis of cDNA from RNA by reverse transcription (RT) firstly, which is then followed with amplification of a specific cDNA by PCR. This is the most useful and sensitive technique for mRNA detection and quantitation that is currently available. RT-PCR is mostly used to detect viruses and the viability of microbial cells through examination of microbial mRNA.

Real-time PCR

In 1993, Higuchi first described a simple, quantitative assay for any amplifiable DNA sequence. This method is based on using fluorescent labelled probes to detect, confirm, and quantify the PCR products as they are being generated in real time. In recent years, some commercial automated real-time PCR systems have been available (LightCycler & TaqMan). In these systems, such as the LightCycler™ and the SmartCycler®, these systems perform the real-time fluorescence monitoring by using fluorescent dyes such as SYBR-Green I, which binds non-specifically to double-stranded DNA generated during the PCR amplification. Others, such as the TaqMan, use florescent probes that bind specifically to amplification target sequences. At present, some broad range primers and probes targeting the 16S rRNA gene have been developed in these systems to detect and identify bacteria universally. The real-time PCR systems not only reduce the detection time (results can be ready in less than on

hour), but also can reduce contamination risks because amplification and detection occur within a closed system.

DNA sequencing

In 1977, two different methods for sequencing DNA were developed, namely, the chain termination method and the chemical degradation method. Both methods were equally popular to begin with, but, the chain termination method soon become more popular and this method is more commonly used today. This method is based on the principle that single-stranded DNA molecules that differ in length by just a single nucleotide can be separated from one another using polyacrylamide gel electrophoresis. The fixed laser beam excites the fluorescently labelled DNA bands and the light emitted is detected by sensitive photodetectors. DNA sequence data is the most accurate and definitive way to identify microbes because the microbes may be identified by base pair to base pair of the nucleic acid. The DNA sequences of the variable regions form the basis of phylogenetic classification of microbes. By sequencing broad range PCR products, it is possible to detect DNA from almost any bacterial species. After comparing the resulting sequences with known sequences in GenBank or other databases, the identity of the unknown bacteria can be revealed. Since the 1990s, 16S rDNA sequencing has become a powerful tool, which is used more and more in microbial detection and identification algorithms, especially for unusual, non-culturable, fastidious and slow growing pathogens, or after antibiotics that have been administered to the patient. Such a technique as this is becoming a routine method of detection and identification of bacteria, thus overall helping to combat infectious diseases.

Gene targets

Unlike diagnostic virology of which targets DNA as well as RNA, traditionally the majority of diagnostic assays in medical bacteriology have been based around the amplification of DNA in a target gene, as opposed to mRNA or other nucleic acid signal. This may be performed as DNA is an extremely stable molecule (Farkas *et al.*, 1996), as opposed to mRNA which has a short half-life (Carpousis, 2002; Steege, 2000). Generally molecular diagnostics in clinical bacteriology is not concerned with regard to the viable status of a organism being detected, but is concerned with the qualitative detection of an organism in a symptomatic patient with the relative clinical presentation, e.g., the detection of meningococcal DNA in the cerebral spinal fluid of a paediatric patient with suspected meningitis. The scenario is completely different where medical bacteriology interfaces with food/public health microbiology, where simple qualitative detection of DNA from pathogenic foodborne bacteria is insufficient or can indeed be misleading and where other more defined molecular viability assays are required, e.g. the molecular detection of viable versus non-viable *Salmonella* sp. in a sample of dried milk powder suspected of causing food-poisoning. In this case, it is insufficient to simply detect the presence/absence of *Salmonella* DNA from an extract of the milk powder as a false positive may be detected under these circumstances, but which in reality represents archival DNA from dead cells killed during the

drying process. Therefore it is important to give careful consideration to what one wishes to achieve from a molecular assay and thus careful emphasis must be placed on the target gene locus.

Universal gene targets

Ribosomal RNA

Where there is no indication regarding the identity of a bacterial organism, employment of amplification of DNA encoding ribosomal RNA genes in conjunction with DNA sequencing of the amplicon has proven to be valuable (Patel, 2001; Kolbert and Persing, 1999). In bacteria, there are three genes which make up the rRNA functionality, i.e. 5S, 16S and 23S rRNA. The 16S rRNA gene has historically been most commonly employed for identification purposes (see Table 1), due to it being highly conserved and having a moderate copy number depending on the genus. 16S rRNA genes are found in all bacteria and accumulate mutations at a slow, constant rate over time, hence they may be used as "molecular clocks" (Woese, 1987). Highly variable portions of the 16S rRNA sequence provide unique signatures to any bacterium and useful information about relationships between them. Since 16S rRNA molecules have crucial structural constraints, certain conserved regions of sequence are found in all known bacteria. "Broad-range" PCR primers may then be designed to recognize these conserved bacterial 16S rRNA gene sequences and used to amplify intervening, variable or diagnostic regions, without the need to know any prior sequence or phylogenetic information about the unknown bacterial isolate.

More recently, employment of the 16S-23S rRNA intergenic spacer region has become popular due to its high copy number and more importantly its high sequence variability (Gurtler and Stanisich 1996; Shang *et al.*, 2003). Primers are directed to highly conserved regions of the 16S and 23S rRNA genes and these may either be universal or specific, targeting a specific genus e.g., *Bartonella* spp. (Houpikian and Raoult, 2001), *Chlamydia* spp. (Madico *et al.*, 2000), *Tropheryma whipplei* (Geissdorfer *et al.*, 2001), *Mycobacterium* spp. (Roth *et al.*, 2000) and *Salmonella* spp. (Bakshi *et al.*, 2002).

Recently, there have been several reports of employment of the large subunit (23S rRNA) which have been used to identify bacterial species. Anthony *et al.* (Anthony *et al.* 2000) reported that the 23S rRNA locus shows more variation between species of medical importance than the 16S rRNA locus. In their study, these workers designed universal 23S rRNA primers which allowed them to detect bacterial agents from 158 positive blood cultures which were identified using a hybridization assay with specific oligonucleotide probes. These workers concluded that the accuracy, range and discrimatory power of their assay could be continually extended by adding further oligonuclotides to their panel without significantly increasing complexity and cost. Furthermore, the 23S rRNA gene locus has been used specifically in order to detect *Stenotrophomonas maltophila* from patients with cystic fibrosis (Whitby *et al.*, 2000).

Table 1. Applications of 16S rDNA PCR to identify causal agents of bacterial infection.

Infection	Clinical specimen	
Bacterial endophthalmitis	Vitreous fluid (VF) and aqueous humour (AH) specimens	Therese *et al.* (1998)
Bloodborne sepsis	Blood-EDTA	Xu *et al.* (2003)
Chronic prosthetic hip infection		Tunney *et al.* (1999)
Detection of tick infecting bacteria		Schabereiter-Gurtner *et al.* (2003)
Endocarditis	Isolate Heart valve Blood culture Blood Arterio-embolic tissue	Woo *et al.* (2003) Moore *et al.* (2001b); Goldenberger (1997) Millar *et al.* (2001) Hryniewiecki *et al.* (2002) Mueller *et al.* (1999)
Endodontic infections		Siqueira *et al.* (2001)
Febrile episodes in leukaemic patients		Ley *et al.* (1998)
Helicobacter sp. osteomyelitis in an immunocompetent child	Biopsy of bone lesion	Harris *et al.* (2002)
Intra-amniotic infection	Amniotic fluid	Jalava *et al.* (1996)
Intra-ocular infection		Carroll *et al.* (2000)
Maxillary sinus samples from ICU patients		Westergren *et al.* (2003)
Meningitis	CSF Blood-EDTA	Saravolatz *et al.* (2003) Xu *et al.* (2003)
Nasal polyps. Chronic sinusitis		Bucholtz *et al.* (2002)
Peritonitis	CAPD fluid from Culture-negative peritonitis	Bailey *et al.* (2002)
Rat bite fever	Blister fluid	Berger *et al.* (2001)
Reactive arthritis	Synovial fluid/tissue	Cuchacovich *et al.* (2002)
Septic arthritis		Van der Heijden *et al.* (1999)
Wound infection	Wound tissue from venous leg ulcer	Hill *et al.* (2003)

Overall, employment of 16S-23S rRNA and 23S rRNA assays have not been as widely used as those targeting the 16S rRNA gene, probably due to there being relatively limited sequence information available for these gene loci in comparison to the 16S rRNA gene, which has been traditionally benefited by there being a formal requirement to describe this rRNA gene in relation to phylogenetic, taxonomic and population genetic studies. Coupled with this, presently the only universal bacterial sequence-based identification scheme available commercially is based on the 16S rRNA gene i.e. the MicroSeq 500 16S ribosomal DNA (rDNA) bacterial sequencing kit (Applied Biosystems, Foster City, CA) (Patel *et al.*, 2000).

Sequence analysis
Sequence-based identification methods employing rRNA gene loci require the use of software to allow the identity of the organism to be made. BLASTn and FASTA software tools are commonly employed to make such comparisons between the query sequence and those deposited in global sequence databases, as outlined in Table 2. Interpretative criteria should be used in order to ascertain the identification of the unknown sequence against its most closely related neighbour (Goldenberger *et al.*, 1997).

Heat shock proteins
Although 16S rRNA may be employed successfully to identify many bacterial species, there are regions within some major genera, in which 16S rRNA gene sequences are not found to be discriminative enough for the identification of certain species, for example, *Burkholderia cenocepacia* and *B. multivorans*. In such circumstances, sequences of essential genes other than the 16S rRNA, such as the heat shock proteins (HSP) (HSP60, HSP65, groEL, groER, etc.), have been shown to be useful (Goh *et al.*, 1996; Woo *et al.*, 2002). The heat shock response is an important homeostatic mechanism that enables cells to survive a variety of environmental stresses. A set of heat shock proteins also known as chaperonins are induced when cells are exposed to higher temperatures. This phenomenon has been observed in all organisms, from bacteria and fungi to plants and animals. The chaperonins are a well-characterized, subgroup of molecular chaperones, which includes the *Gro*E subclass. Heat shock proteins appear to be constituents of the cellular machinery of protein folding, degradation and repair (Feltham and Gierasch 2000). This bacterial molecular chaperone plays an important role in normal growth by mediating the folding and/or assembly of different polypeptides, as well as the transport of some secretory proteins across membranes. For the successful reactivation and assembly of some proteins, *gro*EL requires the presence of another heat shock protein, *gro*ES and the general properties of the heat shock response in many bacteria have been characterized.

Other "universal" gene loci may also be targeted including the *rec*A locus (Matsui *et al.*, 2001) and the cold shock proteins (Francis and Stewart 1997). However, the major disadvantage of employment of these targets is that there is relatively limited sequence data available for comparison of a query sequence against their respective gene sequence databases. For this reason, these targets are not commonly employed or may be confined to identification purposes within a well-defined population, e.g. the *Burkholderia cepacia* complex of organisms (Moore *et al.*, 2001a).

Specific targets
Molecular identification of bacteria can utilise specific gene targets, however in order to do this, prior knowledge of the sequence is required, so that a specific assay may be developed. The advantage of using specific oligonucleotide primers is that they should confer a higher degree of specificity than employing universal or broad-range primers. Examples of specific targets that have been employed include the use of the hippuricase gene for the differentiation of hippurate-hydrolysing campylobacters (*Camp. jejuni*) from non-hydrolysing campylobacters (Slater and Owen 1997) or the use of *ctr*A gene for meningococci in the laboratory diagnosis of meningococcal meningitis (Guiver *et al.*, 2000). The specific target does not necessarily have to be associated with a PCR assay, but may be used in combination with several other nucleic acid amplification/analysis techniques (see Table 3).

Presently, there are several hundred specific assays available for the identification of a diverse variety of bacteria and too numerous to detail in this section. However, the end user of a given assay should be aware that each assay is potentially troubled with several pitfalls of poor assay design, which may lead to poor specificity and/or sensitivity. Therefore, before any assay is adopted into routine diagnostic service, the published method must firstly be empirically optimised in the end user's laboratory and that the user has an appreciation of the strengths and

Table 2. Commonly employed sequence alignment software tools used in conjunction with nucleotide sequence databases.

Sequence identification tools	Nucleotide sequence databases
BLASTn (Basic Local Alignment Sequence Tool) http://www.ncbi.nlm.nih.gov/blast/ (USA) http://dove.embl-heidelberg.de/Blast2/ (Germany) http://www.ebi.ac.uk/blast/index.html (UK) http://www-btls.jst.go.jp/ (Japan)	http://www.ebi.ac.uk/embl/(UK) http://www.ddbj.nig.ac.jp/ (Japan) http://www.ncbi.nlm.nih.gov/Genbank/GenbankSearch.html
FASTA http://www.ebi.ac.uk/fasta33/	
OTHER Ribosomal database project (www.cme.meu.edu/RDP/html/index.html) MicroSeq (Commercial) (www.appliedbiosystems.com) *SmartGene* IDNA (Commercial) (www.smartgene.ch)	

Table 3. Specimen diversity and molecular diagnostic work flow of medically important bacterial infections.

Specimen	Reference
Amniotic fluid	Jalava *et al.* (1996)
Arterio-embolic tissue	Mueller *et al.* (1999)
Ascitic fluid	Such *et al.* (2002)
Atheroma	Apfalter *et al.* (2002)
Blood culture	Millar *et al.* (2001)
Blood-EDTA	Xu *et al.* (2003)
Bone	Harris *et al.* (2002)
Bone marrow	Gamboa *et al.* (1997)
Breast milk	Schmidt *et al.* (1995)
Bronchioelar lavage (BAL)	Ersch *et al.* (2000)
Cerebral spinal fluid (CSF)	Saravolatz *et al.* (2003)
Cervical specimen/tissue	Lehmann *et al.* (1999)
Culture isolate (pure *in vitro*)	Millar *et al.* (2001)
Feces	Kabir (2001)
Fixed tissue sample	Wu *et al.* (2002)
Heart valve	Gauduchon *et al.* (2003)
Lymph node tissue	Yamada *et al.* (2002)
Middle ear fluid	Jero *et al.* (1999)
Nasal polyps/sinus/lavage	Bucholtz *et al.* (2002)
Paraffin-embedded tissue	Yamada *et al.* (2002)
Pus (wound and blister)	Berger *et al.* (2001); Kox *et al.* (1995)
Plasma	Klaschik *et al.* (2002)
Pleural effusion/fluid	Yanagihara *et al.* (2002)
Prosthetic device	Tunney *et al.* (1999)
Saliva	Sakamoto *et al.* (2002)
Semen/sperm	Gdoura *et al.* (2001)
Serum	Such *et al.* (2002)
Skin	Torres *et al.* (2003); Enzensberger *et al.* (2002)
Sputum	McDowell *et al.* (2001)
Swabs	Torres *et al.* (2003)
Synovial fluid	Vander Heijden *et al.* (1999)
Tissue (wound)	Hill *et al.* (2003)
Urine	Mahony *et al.* (1997)
Vaginal fluid	Obata-Yasuoka *et al.* (2002)
Vitreous humour	Sharma *et al.* (2002)
Nucleic acid extraction	
Alkali/heat lysis	Millar *et al.* (2000)
Automated DNA extraction (e.g. MagNAPure)	Raggam *et al.* (2002)
Boil	Clarke *et al.* (2003)
Centrifugation/Chelex-100/boil	Schmidt *et al.* (1995)
Commercial Kits (e.g., Qiagen/Roche)	Millar *et al.* (2000); Clarke *et al.* (2003)
"In house methods"	Millar *et al.* (2000)
Phenol/chloroform	Moore *et al.* (2002)
Silica capture/guanidine hydrochloride treatment/proteinase K/lysozyme	Millar *et al.* (2000)

weaknesses of the assay, so that interpretation of the end result is easier to make.

Antibiotic resistance markers

Recently, antibiotic resistance in bacterial pathogens has become an important topic both nationally and internationally. Some scientists are forecasting the emergence of the "post antibiotic era", where it will be difficult to control common infections, due to the emergence of high level resistance in several medically important bacterial pathogens. Consequently, there has been great interest in being able to detect antibiotic

Table 3. Continued.

	Reference
Nucleic acid amplification/analysis	
Block-based PCR	Tang *et al.* (1997)
– single round	Millar *et al.* (2001)
– semi-nested	Moore *et al.* (2002)
– nested	Hinrikson *et al.* (2000)
– multiplex	Weaver and Rowe (1997)
Branched DNA signal amplification	Tang *et al.* (1997); Zheng *et al.* (1999)
DNA-hybridization/probe assay	Tang *et al.* (1997)
In situ PCR/RT-PCR	Jin and Lloyd (1997)
Ligase chain reaction (LCR)	Blocker *et al.* (2002); Bachmann *et al.* (2002); Wang and Tay (2002); Gilpin *et al.* (2002); Castriciano *et al.* (2002); Rajo *et al.* (2002); Friese *et al.* (2001)
Microarrays	Anthony *et al.* (2001)
Nucleic acid sequence-based amplification (NASBA)	Cook (2003); Cook *et al.* (2002)
PCR-target capture/hybrid capture	Maibach *et al.* (2002)
Q β replicase system	Tang *et al.* (1997); An *et al.* (1995)
Real-time PCR	Klaschik *et al.* (2002)
-Light cycler	O'Mahony *et al.* (2002)
-Taqman	Ellerbrook *et al.* (2002)
Reverse transcriptase PCR (RT-PCR)	Tang *et al.* (1997)
Strand displacement amplification (SDA)	Ge *et al.* (2002)
Transcription mediated amplification (TMA)	Hill (2001)
Characterization/Identification of amplicon	
Automated sequence analysis	Patel (2001)
DNA-DNA hybridization	Siqueira *et al.* (2001)
Enzyme immunoassay (PCR-EIA)	Moreno *et al.* (2003)
Restriction enzyme analysis (PCR-REA)	Brown and Levett (1997)
Restriction fragment length polymorphism (RFLP)	McDowell *et al.* (2001)
Single-strand conformational polymorphism (SSCP)	Kerr and Curran (1996); Hein *et al.* (2003)
Genotyping/Molecular Epidemiology	
Amplified fragment length polymorphism (AFLP)	Moreno *et al.* (2002)
Arbitrary-primed – PCR (AP-PCR)	Dabrowski *et al.* (2003)
Multilocus sequence typing (MLST)	Enright and Spratt (1999)
Pulsed field gel electrophoresis (PFGE)	Wu and Della-Latta (2002)
Random amplification of polymorphic DNA-(RAPD)	Power (1996)
- BOX PCR	Gillespie (1999)
- ERIC PCR	Marty (1997)
- rep PCR	Baldy-Chudzik (2001)
Ribotyping	Moore *et al.* (2002a)
Single-strand conformational polymorphism (SSCP)	Kerr and Curran (1996); Hein *et al.* (2003)

resistance molecular, particularly when the causal agent is fastidious or non-culturable. Fluit *et al.*, (2001) have recently published an authoritative review on the molecular detection of antibiotic resistance. Currently, most hospitals are concerned with the occurrence of methicillin resistance *Staphylococcus aureus* (MRSA) and glycopeptide resistant enterococci (GRE) on their wards, particularly surgical wards. Several workers have published molecular methods to detect MRSA through employment of a simple PCR assay, targeting the *mec*A gene locus (Kobayashi *et al.*, 1994; Towner *et al.*, 1998). The employment of *mec*A PCR to screen for carrier status of patients in the intensive care unit (ICU) is a useful tool to allow infection control teams to segregate positive MRSA patients from non-colonised patients, thereby minimising the opportunity for nosocomial spread amongst ICU patients. Application of such an assay also demonstrates that for this screening policy to be effective, the host laboratory should be in a position to perform the assays locally and not rely on sending cultures to a Reference laboratory.

Genomovar analysis of *Burkholderia cepacia* in cystic fibrosis
Infection with the *Burkholderia cepacia* complex (BCC) is an important cause of increased morbidity and reduced

survival in patients with cystic fibrosis (CF) (Høiby 1991). Certain members of the BCC are transmissible and epidemics have been described in a number of CF centres (Doring *et al.*, 1996). A number of factors such as the presence of the *B. cepacia* epidemic strain marker (BCESM) (Mahenthiralingam *et al.*, 1997) and the cable pilus gene (Sajjan *et al.*, 1995) have been identified as markers of transmissibility. Most units in the UK now segregate patients with this infection from all other CF patients and infection control guidelines have been recently published by the UK CF Trust (Anon., 1999) to help reduce the potential for cross infection with these organisms. Nine genomovars of the BCC have now been formally described and initial studies indicate that genomovar II has less clinical impact than genomovar III (De Soyza *et al.*, 2000). Presently *B. cenocepacia* (formerly genomovar III) followed by *Burkholderia multivorans* (formerly *B. cepacia* genomovar II) make up the majority of patients infected with BCC. Some centres now segregate patients with different genomovar types eg., genomovar III patients from other *B. cepacia* complex infected patients (e.g. Belfast and Vancouver) to reduce the risk of cross infection between patients with BCC. Presently some centres believe that complete segregation of all the genomovar types is the best policy, whilst others feel that it is only important to segregate genomovar III from the other BCC types. Early accurate identification of BCC is of critical importance so the patients can be segregated and therefore reduce the potential for further epidemics. BCC organisms present the clinical microbiologist with a diagnostic dilemma, in that there are extremely few and in some cases no phenotypic biochemical or growth-related characterization tests that reliably distinguish between these organisms. To this end, there have been a variety of different molecular-based characterization tests to differentiate the genomovars (Segonds *et al.*, 1999; LiPuma, 111999). Recently, the *rec*A gene has been shown to aid in the differentiation of the genomovars (Mahenthiralingam 2000). RecA is a multifunctional and ubiquitous protein involved in general genetic recombination events and in DNA repair. It regulates the synthesis and activity of DNA repair (SOS induction) and catalyses homologous recombination and mutagenesis. As this locus may contribute significantly to the overall genomic plasticity of the BCC, this locus is potentially a strong candidate for the adoption of rapid molecular-based assays in the laboratory. Furthermore, molecular methods may aid CF centres in helping determine the status of CF patients with regard to whether they are chronically, transiently or not colonised with this pathogen.

Molecular diagnostic procedures

Most molecular assays rely on three basic components, including nucleic acid extraction, amplification/analysis and detection of an amplified product, as outlined in Table 3. Most molecular assays allow for a wide variety of permutations and combinations of methods, depending on what is trying to be achieved. For example, almost all clinical specimen types have been extracted by a variety of DNA extraction protocols. Choice of nucleic acid amplification/analysis may be varied, however presently,

application of "real-time" platforms including the Roche LightCycler and the ABI Taqman 7700 systems are popular, as these not only allow detection in a relatively short time in a gel-less system, but also for the quantification of copy numbers. There are several factors which help determine which type of assay to employ (see Table 4). If speed is an important factor of the assay, e.g. detection of meningococcal DNA in CSF from children with suspected meningitis, employment of the real-time assays should be adopted. Where numerous targets are important, the multiplex PCR format should be employed.

Laboratory management of molecular assays

All molecular assays, due to their high sensitivity, are prone to contamination problems, which has the potential to lead to false-positives, thus diminishing the effectiveness of such testing regimes and thus strict contamination controls need to be established to avoid the occurrence of such problems. The use of broad-range PCR is particularly susceptible to contamination problems (Millar *et al.*, 2002) and it has also been argued that such problems are indeed not unique to broad-range PCR, but that can be equally applied to specific PCR (Bastien *et al.*, 2003). From clinical specimen reception to molecular analysis, there are numerous sources of contamination risk and for each molecular assay these should be identified and appropriate control measures identified and established to minimize each risk (see Table 5 for working example). To ensure the minimum contamination risk, including PCR amplicon carry-over, a key element is successful workflow through geographically separated areas. It is recognized that many diagnostic facilities in hospital laboratories have limited work space or have poor quality work space that makes separation of pre- and post-PCR areas difficult to achieve. However, it is important that adequate space is allocated to ensure compliance and to conform to CPA Accredittion standards for molecular diagnosis (Anon., 1999).

Successful employment of PCR in the detection of causal agents of infectious disease is critically dependent on both the quantity and quality of controls associated with the assay, to avoid the occurrence of both false-positive and false-negative results. Table 6 lists the reasons for false-positive and false-negative findings. It should be noted that for each PCR-based test, sensitivity should be evaluated for each specimen type, prior to routine implementation. It is vital that several negative and positive controls are set up during each diagnostic run. Negative and positive controls should include (a) DNA extraction control, (b) PCR set up control and (c) PCR amplification control. For DNA extraction purposes, the positive control should include clinical specimen artificially spiked with organism, e.g. blood culture spiked with *E. coli*. For clinical tissue specimens where a true positive specimen may be difficult to mimic, internal positive controls may be employed, such as following DNA extraction, amplification of the β-globin gene, as previously described (Millar *et al.*, 2001). With respect to PCR controls, the positive control should be bacterial DNA extracted from a pure culture. Ideally, the positive control should include two components, namely: (i) a specimen generating a weak signal, due to low copy numbers of target and (ii) a specimen generating

Table 4. Criteria used in the determination of the most appropriate molecular method for use in bacterial infections.

Criterion	Appropriate molecular method of choice	Comment/example
Time/speed to detection	Real-time applications (LightCycler/TaqMan 7700 system)	Detection of meningococcal DNA from clinical specimens in children with suspected meningitis (Guiver *et al.* 2000)
Quantification	LightCycler/TaqMan 7700 system/NASBA	Determination of effect of antibiotic intervention
Multiple targets	Multiplex PCR/microarrays/hybridization probe assay	Determination of multiple respiratory pathogen from sputa
Viability	NASBA/RT-PCR	Determination of viable pathogens in foodstuffs or detection of viable but non-cultural (VNC) organisms (Cook, 2003)
Commercial availability	MicroSeq (ABI Ltd.), LCR (Abbott)	Identification by comparison of query organism with high quality database (4). Detection of *Chlamydia* in genital specimens (Bachmann *et al.*, 2002, Castriciano *et al.*, 2002, Friese *et al.*, 2001)
Throughput	High – Real-time PCR Low – Block-based PCR	Automated DNA extraction followed by real-time PCR

Table 5. Potential sources of DNA contamination and its remediation measures in molecular diagnostic analyses.

	Specimen collection	Nucleic acid extraction	PCR analysis
	Type: blood *Vial*: EDTA *Location*: hospital ward *Personnel*: nurse, junior doctor	*Method*: Commercial Kit *Additional reagents*: lysozyme, water, Tris-HCl *Location*: class II biological safety cabinet *Personnel*: MLSO	*Reagents*: water, buffer, MgCl$_2$, dNTPs, Taq, primers *Location*: PCR cabinet in different location to nucleic acid extraction and post PCR *Personnel*: MLSO
Contamination risk	1. Commensal flora on patient's skin 2. Commensal flora on staff's skin 3. Archival DNA in specimen vial 4. Archival DNA in EDTA solution 5. Inappropriate collection of specimen by personnel	1. DNA from kit reagents 2. DNA from additional reagents 3. Location 4. Class II biological safety cabinet 5. Pipettes 6. Equipment e.g., centrifuge, heating block, vortex 7. Reaction vials (1.5ml) 8. Personnel	1. DNA from reagents 2. Location 3. PCR cabinet 4. Pipettes 5. Equipment e.g., vortex 6. Reaction vials (1.5ml) and PCR tubes 7. Personnel
Control actions to eliminate exogenous contamination	1. Iodine scrub of puncture site 2. Wearing of sterile gloves and protective clothing 3/4. Employment of laboratory prepared and quality controlled DNA-free blood-EDTA vials 5. Education of personnel	1/2. All reagent purchased should be of molecular grade. All reagents should be screened prior to use. Contaminating DNA should be monitored using negative DNA extraction controls 3. A dedicated pre-PCR room should be used, ideally under positive pressure Unidirectional work flow 4. Cabinet should be cleaned thoroughly and UV irradiated for a minimum period of 2h prior to use Cabinet should be serviced regularly 5. Dedicated pipettes should be used and should not be removed from the class II cabinet. Plugged sterile tips should be employed throughout. Pipettes should be cleaned and the barrels UV irradiated for a minimum of 2h prior to use 6. Equipment should be dedicated to extraction purposes only 7. Reaction vials should be pre-autoclaved and DNA-free. 8. Education of personnel. Use of sterile gloves and dedicated laboratory clothing for DNA extraction purposes only 9. Avoidance of re-usable laboratory glassware	1. All reagents purchased should be of molecular grade. All reagents should be screened prior to use. PCR master mix minus the primers and template DNA should be UV irradiated for 15 min prior to amplification. Contaminating DNA should be monitored using negative PCR setup controls 2. A dedicated pre-PCR room should be used, ideally under positive pressure and separate from DNA extraction procedures. Unidirectional work flow 3. Cabinet should be cleaned thoroughly and UV irradiated for a minimum period of 2h prior to use. Cabinet should be serviced regularly 4. Dedicated pipettes should be used and should not be removed from the PCR setup cabinet. Plugged sterile tips should be employed throughout. Pipettes should be cleaned and the barrels UV irradiated for a minimum of 2h prior to use 5. Equipment should be dedicated to PCR setup purposes only 6. Reaction vials and PCR tubes should be pre-autoclaved and DNA-free. 7. Education of personnel. Use of sterile gloves and dedicated laboratory clothing for PCR setup purposes only. 8. Avoidance of re-usable laboratory glassware

Table 6. Most common factors contributing towards false positive and false negative results in molecular diagnostic assays.

Reasons for false-positive results	Reasons for false-negative results
Carry over contamination (amplicons) from previously amplified products	Inhibition of PCR reaction
Presence of exogenous target DNA in reagents, water, kits, sterile blood culture material	Inadvertent loss of template nucleic acid target due to poor extraction, handling and storage protocols
Poor primer design (non-specificity)	Digestion of nucleic acid template with endogenous DNAses and RNAses
Inadequate amplification conditions	Poor primer design (non-conserved regions at primer site(s) in variants)
Contamination from laboratory personnel	Poor intrinsic sensitivity of nucleic acid amplification/analysis detection system Poor sensitivity of nucleic acid amplification/analysis reaction Poor specificity Inadequate amplification conditions

a strong signal, due to high copy numbers of target. By employing these controls especially the negative controls, it is easier to identify the point of contamination within the diagnostic assay e.g., DNA extraction contamination free but contaminated at PCR set up stage. Positive controls are also important, particularly those included in the DNA extraction procedures, as they serve to identify possible inhibition of the PCR reaction, due to inhibitory agents in the biological specimen, which co-elute with extracted DNA, e.g. sodium polyanetholesulfonate in blood culture material (Millar *et al.*, 2001). For a comprehensive review on PCR inhibition with respect to biological specimens, see Wilson (Wilson 1997).

Standardization and harmonization
Presently there are numerous molecular methodologies for the identification and genotyping of both culturable and non-culturable bacterial causal agents of infection. Although various commercial approaches have been described, such as the MicroSeq PCR-DNA sequencing identification system, the majority of methods used in clinical microbiology laboratories are "in-house". To date, there has been little or no attempts made to standardize and harmonize bacterial detection protocols between laboratories at a local, national and international level, although several European centres have attempted to examine these for specific organisms (Struelens and others 1996; Deplano *et al.*, 2000; Dijkshoorn *et al.*, 2001; Fry *et al.*, 1999; Grundmann *et al.*, 1997; Van Belkum *et al.*, 1998). The disadvantage of not employing such standardized methods may lead to anomalies in the epidemiologically, which may yield bias results and hence corrupt the epidemiological reporting of a variety of infectious disease states. Most diagnostic laboratories employ methods, which are not completely consistent with their peer laboratories, e.g. employment of a published method with variations in the empirical optimization and/or with different reagent suppliers. Presently, there are limited studies detailing the effect of such variation on qualitative reporting of results and hence this area requires urgent attention. More recently, various focussed attempts have been made to try to standardize bacterial subtyping techniques, through the actions of PulseNet (http://www.cdc.gov/pulsenet/), primarily for bacterial foodborne disease surveillance in the US and the ESF Network for Exchange of Microbial Typing Information European Network (ENEMTI) (http://lists.nottingham.ac.uk/mailman/listinfo/enemti). ENEMTI is a network of European laboratories that aims to standardize methods and data exchange protocols for internet-based comparison of microbial fingerprinting data. This project aims to develop an internet-based database system for DNA fingerprints that is readily accessible and user-friendly for microbiologists with only limited computer expertise. In addition, the European Society for Clinical Microbiology and Infectious Disease (ESCMID) have a specific working group, namely the ESCMID Study Group on Epidemiological Markers (ESGEM), whose objectives are to (a) critically evaluate microbiological typing systems and make recommendations for their appropriate use, (b) promote collaborative research into microbiological typing systems and to develop standardized methodology for specific pathogens, (c) provide a forum for the exchange of ideas and the development of consensus strategies and (d) work with individuals and companies active in this research area to foster the development of further technological advances in microbial typing (http://www.escmid.org/sites/index_f.asp?par=2.5).

Appraisal of molecular diagnostics in clinical bacteriology
Molecular diagnostics have several advantages and disadvantages for their adoption into routine bacteriology, as detailed in Table 7. At present, employment of molecular diagnostics is largely confined to specialized or reference bacteriology laboratories, due to several factors. With such an approach, molecular methodologies including PCR, real-time PCR and PFGE may have the opportunity to become adopted by several regional diagnostic laboratories outside of highly specialized reference facilities, as such methodologies have been shown to provide valuable real-time information to aid in outbreak management and identification of non-culturable or fastidious organisms. To date, the speed in which these assays have been taken up has been closely related to the relative skills base within the laboratory, thus although there is an advantage in all hospital types utilising such technology i.e. from the district general to university teaching hospitals, such techniques have not become common where the skills base does not exist. Furthermore, there is the added danger that hospital research facilities, which have been the custodians of these research techniques to date, become overloaded with a molecular diagnostic workload, simply because they have the necessary skills base. In addition, molecular techniques are perceived to be relatively expensive, which is the case on a simple head-to-head comparison (Table 8). The overall value of the quality of the test result,

Table 7, Advantages and disadvantages of the adoption of molecular assays into clinical bacteriology.

Diagnostic criterion	Advantages	Disadvantages
Accuracy of identification	Aid in identifying aetiological agents of infections which are difficult to culture, including: Negative cultures Expensive cultures Slow growing organisms Fastidious organisms Cell-dependent organisms Category 3 cultures where a designated secure cell culture laboratory is required Difficult, specific culture requirements where limited serological tests exist identification of causal agent following antibiotic therapy	Problems associated with contaminant organisms, however these problems may be aided by inclusion of appropriate DNA extraction and PCR controls (Millar *et al.* 2002) The agent identified should be considered with respect to the patient's medical and general history
Time to detection Where specimens are: a. culture-positive and/or serology-positive b. culture-negative and/or serology-positive	Confirmation of conventional detection result more rapid detection than conventional culture for fastidious and cell-dependent organisms Confirmation of serology result. Detect and highlight non-specific serological false-positive results	Longer time required for molecular PCR and sequence analysis than culture & serology for non-fastidious or cell-dependent organisms Longer time required for molecular PCR and sequence analysis than serology
c. culture-negative and serology-negative	Identification of causal agent when all conventional diagnostic assays are negative	
Impact on therapy	Appropriate antibiotic therapy can be commenced sooner or modified earlier in the presence of a molecular identification from a culture-negative/serology-negative specimen Provision for PCR detection of antimicrobial resistance gene determinants in culture-negative PCR-positive specimen	
Cost-effectiveness	Cost-effective particularly with culture-negative/serology-negative specimens to avoid extended analysis for several potential pathogens either by specific culture and serological testing. Economic and early use of most appropriate cost-effective antibiotic treatment regimen Economic and optimised in-patient stay	Not cost effective when conventional culture and serology give quality and early identification result Specialised equipment Necessary to purchase/lease specialised equipment usually with costly maintenance contracts Space allocation Lack of education in modern molecular based technologies Medical laboratory scientific officers, clinical scientists and medical microbiologists all must understand the principles of molecular based technologies to ensure proper handling of the specimens and appropriate interpretation and significance of results (Moore and Millar 2002), hence specific training must be given

Table 8. Comparison of financial cost of identification of a bacterial culture employing phenotypic and genotypic identification schemes.

Routine/conventional (API) identification		Molecular (16S rDNA PCR & sequencing) identification	
Consumable item	Approx. cost (GBP £) (ex VAT)	Consumable item	Approx. cost (GBP £) (ex VAT)
1 x API20NE strip	3–95	DNA extraction kit	2–50
API diagnostic reagents[a]	0–80	PCR reagents (not including primers)	0–12 (Taq) + 0–14 (dNTPs)
		Gel electrophoresis	1–09
		Plasticware consumables	1–50
		Specialist reagents (TAE, Tris, EtBr, etc)	0–10
		PCR primers (forward and reverse)	0–10
		PCR sub-total costs	5–55 x 2 = 11–10
		Sequencing kit	1–96
		Polyacrylamide gel[b]	0–93
		Plasticware consumables	1–50
		Sequencing primers (Cy-5' labelled)	0–20
		Sequencing sub-total costs	4–59 x 2 = 9–18
TOTAL	4–75		20–28

[a]Assuming one set of reagents are adequate for two kits, [b]assuming the amplicon is sequenced as part of a ten-set batch.

however, should be taken into account in terms of several parameters, including time-to-detection and ability to detect an agent. Although the use of a molecular test is albeit more expensive, its employment may yield a finding that could potentially reduce costs significantly further downstream with patient management.

In conclusion, molecular diagnostic techniques have a significant role to play in clinical bacteriology, although their adoption will never replace conventional methodologies, which continue to be the cornerstone of modern bacteriological methods. Indeed, such molecular diagnostic assays may only be implemented in specialized laboratories to enhance laboratory diagnostic efficiency, where the use of such assays will be mainly confined to diagnosis, identification and genotyping, where current conventional approaches are grossly inadequate. Adoption of such methods in bacteriology has occurred at a much slower rate than in clinical virology, where the inadequacies of conventional virology, has accelerated the adoption of molecular methods. Integration of molecular approaches in clinical bacteriology will be enhanced through the production of a greater range of diagnostic kits, as well as the existence of more accredited laboratories.

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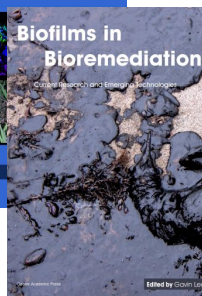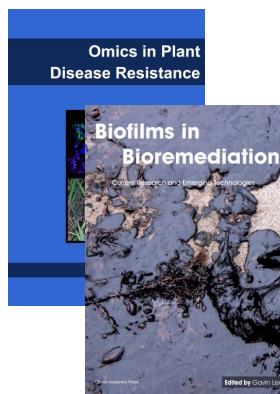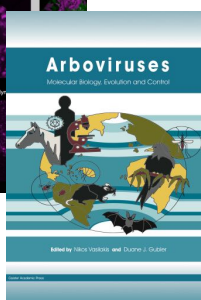
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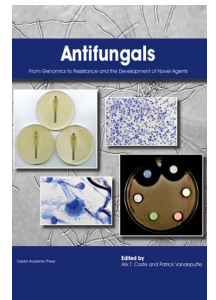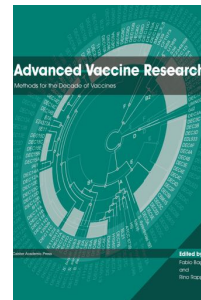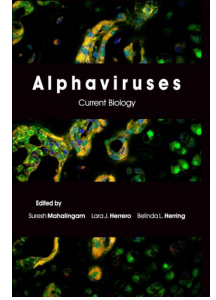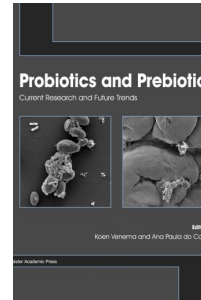
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