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Chapter 30

Molecular Diagnostics and Comparative Genomics in Clinical Microbiology

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30.1 INTRODUCTION

Invasive infections in humans can be caused by a wide variety of protozoan, fungal, microbial, and viral pathogens. This renders microbiological and viral diagnosis a complicated task, especially since many of these pathogens generate similar and sometimes even identical clinical syndromes. As a further complicating factor, clinical samples submitted for culture-based diagnostic procedures are frequently contaminated by microorganisms naturally colonizing the epithelial lining of the human body. Moreover, the specimens also may contain substances that limit the chances for pathogen's survival and, thereby, cultivation success. On the whole, microbial diagnostics is usually performed on a wide variety of clinical samples and the microbial agents requiring identification may differ in prevalence with age, sex, habits, demographics, health status, and several other personal characteristics of the individual patient. The prevalence of infectious agents may also be subject to strong seasonal and geographic variation and is currently considered to be strongly influenced by travel habits as well. In all, culture-oriented clinical microbiology seems to be an art rather than a precise scientific approach. With respect to correctness of a diagnosis, much depends on the expertise of the clinical microbiologist or infectious disease (ID) specialist: during the diagnostic procedures several important decisions must be taken and these may be biased due to personal preferences rather than scientific appropriateness.

The choice of microbiological laboratory procedure is usually dictated by personal experiences: doctors ask for a specific type of diagnostic assay, but this may not always be the most obvious one. After clinical parameters have been assessed at bedside, the first step in microbial diagnosis normally consists of a straightforward (gram-) staining procedure. This segregates gram-positive from gram-negative bacteria and already, in part, may lead to direct species identification of the pathogen involved. Malaria parasites, for instance, show very distinct morphological features upon simple Giemsa staining of a blood smear (Iqbal *et al*., 2003). Subsequently, the clinical specimen may be further analyzed by cultivation-based tests. In addition, concomitant host serum samples may be analyzed for antibodies raised by the host's immune system against the pathogen involved. Microbial antigens rather than the complete and viable organism itself can be searched for as well. Finally, when investigations are finished and a pathogen has been identified beyond any reasonable doubt, rational therapy can be implemented, if available. Therapy is usually preceded by assessment of the antimicrobial susceptibility profile and/or virulence characteristics of the microorganism involved; that is, in case of successful cultivation, of course. It goes without saying that speed and quality of the diagnostic procedures in the end determine the clinical impact and efficacy of antibiotic therapies.

Since some pathogens may be transmitted easily between humans, not only diagnosis is important. It is also relevant to keep track of the spreading of certain pathogens, both in the hospital and in the open population. Classic microbiology uses methods such as phagetyping, serotyping, or antibiogram comparison to assess epidemiological relatedness among strains of a given pathogenic species. The diagnostic and epidemiological methods just mentioned have been in place for already quite some time. Seeing the continuing impact of infectious diseases on modern society (i.e. the AIDS pandemic, biological warfare, emergence of multidrug resistant bacteria, the recent SARS outbreak), there is a persisting need for continuous improvement of diagnostic microbiology and virology (Demain, 1999). Initially, serological tests, covering both

host antibody responses assessment and bacterial serotyping, helped improve adequate diagnosis of several infectious diseases. More recently, molecular microbiology has provided the laboratory with additional tools that will help significantly to improve the quality of microbial detection and (subspecies) identification (see Fig. 30.1).

Molecular microbiological techniques initially were developed during the 1960s and 1970s in fundamental research laboratories. As a direct result, over the past one or two decades, the identification of the organisms that are infectious to humans has been greatly facilitated by the development and application of specific molecular hybridization (probe) tests (Ksiazek *et al*., 2003). In addition, the availability of (real-time) nucleic acid amplification methods has been instrumental in the development of another, even more recent category of direct, highly sensitive diagnostic assays. The polymerase chain reaction (PCR) is among the most popular of these methods (Wolk *et al*., 2001). The currently available molecular assays enable both the direct detection of antigens (DNA and RNA) of putative pathogens in clinical material and the genetic identification (also known as DNA fingerprinting or comparative genomics) of microorganisms obtained by culture. Fingerprinting also allows for species identification and, more importantly, facilitates the identification of molecular markers that are associated with the disease invoking potential of a given strain of a potentially pathogenic microorganism. So-called pathotyping is an important recent development.

The availability of molecular diagnostics initially was considered a panacea, but replacement of conventional tests for detection and identification of microorganisms by molecular procedures turned out to be a slow and cautious process. However, several of the innovative nucleic acid identification tests are currently just beyond or slightly ahead of their breakthrough. For all the clinically relevant microorganisms one or more molecular tests for detection and identification, even below the species level, have now become available, although commercially developed tests are for sale for only the most prevalent infectious disease agents. High-throughput use of these tests, however, is becoming more common although still somewhat restricted to the larger laboratories possessing adequate technical equipment and analytical expertise.

In this chapter, the current state-of-the-art of molecular diagnostics and comparative genomics in medical microbiology will be described, together with the technological advances that have been made recently. In addition, some of the problems remaining to be solved prior to general acceptation of nucleic acid-mediated detection and identification of microbial pathogens will also be reviewed. This chapter highlights the success of the novel applications by providing examples of modern molecular diagnostic approaches in the field of bacterial infections. Viral diagnosis will not be discussed in depth, but a short section describing the current state of affairs within virology will be provided later in this chapter.

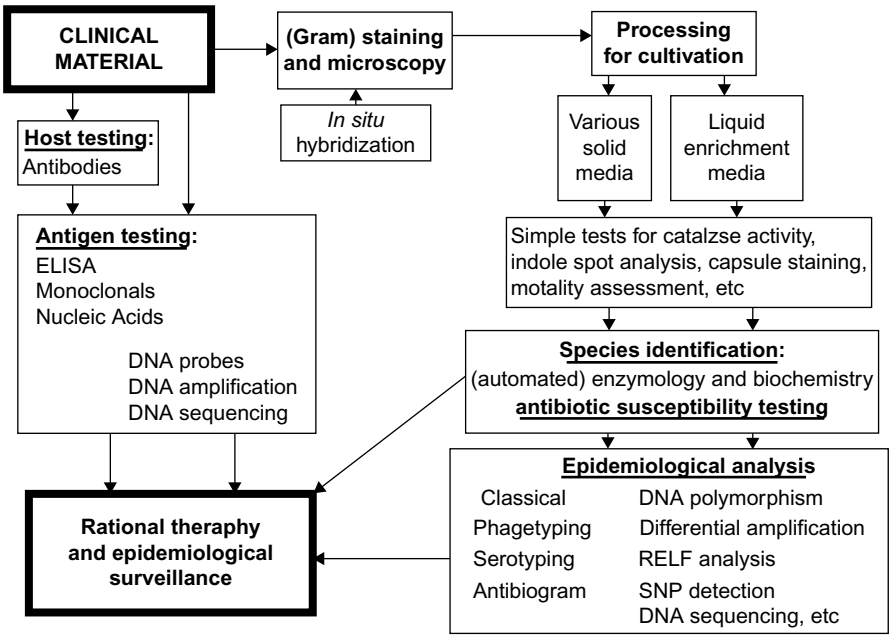

FIGURE 30.1 Schematic representation of sample routing in clinical microbiology. The classic methods are depicted in standard letter type, whereas the novel molecular diagnostic tools are depicted in italic and bold lettering. The arrows indicate the flow of activities; note that epidemiological investigations can be guided by data from both the antigen detection and culture-based test systems (ELISA: enzyme-linked immunosorbent assay; RFLP: restriction fragment length polymorphism; SNP: single nucleotide polymorphism).

30.2 TECHNOLOGICAL IMPROVEMENTS

Essentially, molecular diagnostics of infectious diseases is simple and straightforward. A clinical sample (or a not yet identified organism derived from a microbiological culture experiment) is provided and the use of a broad-spectrum nucleic acid isolation procedure generates material to be included as a template in nucleic acid hybridization or amplification assays. These reactions proceed and positive or negative results are produced, interpreted, and translated into a diagnostic result. However, this simple scenario has been compromised by a variety of obvious and emerging problems. Most of these have been solved, but some remain and are continuously investigated in search of elegant solutions. Some examples of recent technological successes follow.

30.2.1 Optimization of the Isolation of Template Nucleic Acid Molecules

During the past decade, the guanidinium isothiocyanate/Celite affinity procedure was accepted as the gold standard in many molecular diagnostic laboratories (Boom *et al*., 1990). Its main downside was the fact that the manual version of this procedure was and still is quite time-consuming and laborious. Fortunately, alternative systems have become available. Extraction simplification and automation have progressed well and after the commercial availability of handy spin column assays (e.g. the Qiagen kits; McOrist *et al*., 2002), fully automated nucleic acid isolation systems have been developed. bioMérieux has automated the Celite affinity procedure through the Nuclisense Extractor machine. Roche Molecular Systems is marketing the MagnaPure DNA isolation robot, which allows for parallel DNA extractions for hundreds of samples per working day, invoking limited hands-on time only (Van Doornum *et al*., 2003). In a recent study, three methods (NucliSens miniMAG (bioMérieux), MagNA Pure DNA Isolation Kit III Bacteria/Fungi (Roche), and a silica-guanidiniumthiocyanate (Si-GuSCN-F) procedure for extracting DNA from stool specimens) were compared. The manual Si-GuSCN-F procedure showed the highest analytical performance at the lowest associated costs per extraction (euro 4.28). However, this method did require the longest hands-on time. Both miniMAG and MagNA Pure extraction showed similar performances. With regard to amplification inhibition all methods showed relatively low rates (<4%). Costs, finally, were euro 4.28 for Si-GuSCN, euro 6.69 for MagNA Pure, and euro 9.57 for miniMAG (Schuurman *et al*., 2007). Further improvements in the nucleic acid yield and sample throughput are anticipated. The currently separate DNA extraction and amplification machines will soon be combined in a single apparatus (Cockerill and Smith, 2002).

To monitor the extraction process for loss of target or contamination by inhibitory compounds, internal process controls should be introduced (see also section 30.4 for more details). This methodology identifies deleterious effects during extraction (loss of sample) and amplification (inhibition), and enables one to be more confident on both positive and negative results generated. It has to be stated that molecular microbiologists have been putting in enormous efforts to demonstrate the efficacy of nucleic acid-mediated testing over the past years. Far more than classic microbiology, DNA/RNA testing has been subjected to extremely detailed and stringent intercenter quality control studies. Interestingly, most of the new generations of tests withstood the comparisons with classic testing quite easily, thereby confirming their superiority over the widely accepted classic test systems (Harmsen *et al*., 2001; Niesters, 2002). Recently, the molecular diagnostics of methicillin resistant *Staphylococcus aureus* MRSA bacteria has also been the subject of such a multicenter validation study (van Belkum *et al*., 2007a).

30.2.2 Prevention of Carry-Over Contamination

The implementation of PCR and other amplification tests in routine laboratories requires logistic adaptation and training programs for the personnel involved. For one, the various stages in PCR diagnostics (i.e. nucleic acid purification, preparation of enzyme master mixes, collating the complete PCR samples, amplification, and, finally, the analysis of the amplified material) should preferably be performed in physically separate laboratories. It is advisable even to plan personnel involvement in a downstream manner: individuals that have been in contact with amplimers should not be allowed to enter the clean laboratory anymore that day. The most important adaptations are those required to prevent contamination and subsequent false positive results. The current generation of commercial assays uses intelligent systems such as the UNG glycosylase approach (Meier *et al*., 1993). This results in pre-PCR destruction of amplimers generated in prior tests; this system has proven to be quite robust (Rys and Persing, 1993). There are several other measures and precautions to be taken that essentially prevent carry-over contamination from occurring. Replacement of DNA-contaminated disposable articles by clean ones, avoiding pipette abuse (aerosol formation!), adequate routing of patient materials, and the use of molecular-grade PCR ingredients are a few of these alternative options. A comprehensive discussion of most of the important factors was recently published by Millar and colleagues (2002).

30.2.3 Real-Time PCR Systems

The initial molecular tests for infectious diseases were strictly qualitative in nature: they provided a straightforward

yes-or-no answer to questions relating to the absence or presence of certain pathogens. With recent improvements of the nucleic acid-mediated technology, quantitative aspects also became addressable. Current methodologies allow for the assessment of the relative quantity of a given nucleic acid target molecule in a clinical sample (Barken *et al*., 2007). A variety of real-time PCR machines have been developed. These include, among others, the GeneAmp 5700 and Prism 7700 by Applied Biosystems, the BioRad iCycler, the Roche LightCycler, the Cepheid Smartcycler and GeneXpert, the MX400 by Stratagene and the Rotor Gene by Corbette Research (see also Chapter 7). All these machines share a high assay speed (between 20 minutes and 2 hours per run) and sufficient sample capacity (from 16 to 384 samples per run). Real-time technologies combining PCR and TaqMan hydrolysis probes (Lunge *et al*., 2002), NASBA and molecular beacons (Weusten *et al*., 2002), or hybridization probes (Bidet *et al*., 2003) helped to improve the tedious process of real-time detection and identification. It has been well established that the new technologies have the reproducible ability to detect (small amounts of) an infectious agent in all sorts of clinical specimens.

The question whether nucleic acid quantification may have an added value for clinical infectious disease management in distinct groups of patients should and must be answered in the near future. The main issue here is whether larger amounts of pathogens present may be an indication of the severity of (underlying) disease. If this is the case (as suggested by some virology studies; Schutten and Niesters, 2001) this implies that quantitative tests could also be used successfully to monitor the efficacy of a treatment protocol.

30.2.4 Broad Spectrum PCR and PCR Multiplexing

In some cases, species of microbial pathogens capable of invoking an infectious syndrome may be large in number. Infection of the lower and upper airways, for instance, can be caused by a variety of bacterial species. These include, in random order, *Streptococcus pneumoniae*, *Moraxella catarrhalis*, *Haemophilus influenzae*, *Bordetella pertussis*, *Mycoplasma pneumoniae*, *Legionella pneumophila*, *Chlamydia pneumoniae*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and others covering a wide variety of viruses, fungi, and parasites as well. It would be very cost effective if these pathogens, involved in similar, clinically manifest diseases, could be detected by a single assay. Broad spectrum PCR coupled to species-specific nucleic acid probing assays could provide such an opportunity (Roth *et al*., 2000), whereas multiplex PCRs have been described that are capable of achieving a similar feat (Ko *et al*., 2003). This type of assay will become increasingly important over the coming years, especially with the introduction of quantitative variants of these tests. The downside of multiplexing is that the multiplex PCR is usually considered to be less sensitive than the simplex approach. This problem could be circumvented by applying a new generation of PCR-mediated tests. For instance, the multiplex ligation probe assay is supposed to be relatively insensitive to the downside of classical PCR multiplexing (Reijans *et al*., 2008). A recent test was described which supposedly detects 15 respiratory viruses in one reaction. The MLPA reaction is preceded by a pre-amplification step which ensures the detection of both RNA and DNA viruses with the same specificity and sensitivity as individual monoplex real-time RT-PCRs. The use of this test showed an increase of the diagnostic yield from 35.5 up to 60% compared to cell culture. The test provided a user-friendly and high-throughput tool for the simultaneous detection of 15 respiratory viruses with excellent overall performance statistics.

The detection of ill-defined or even previously unrecognized bacterial pathogens can be pursued by broad spectrum PCR, a method already alluded to in the previous section (Relman, 1998). The method usually involves PCR targeting of universally conserved, eubacterial PCR priming sites. A well-known example of such targets are the ribosomal genes, although successful broad spectrum amplification of genes encoding DNA polymerase, DNA gyrase, or protein elongation factors has been described as well. Broad spectrum PCR has recently been used to try to resolve a variety of clinical syndromes. These include, for instance, periodontal diseases (Kumar *et al*., 2003) and non-chlamydial, non-gonococcal urethritis in men (Riemersma *et al*., 2003). The latter study elegantly demonstrated that in infectious diseases not only the presence of a pathogen may be important, but also the absence of certain apparently healthy components of the resident flora may be helpful in diagnosing disease. There may be, however, fundamental obstructions to the diagnostic implementation of broad spectrum PCR. For instance, there is an intense discussion still ongoing on the presence of native, non-pathogenic commensal bacteria in human blood (Nikkari *et al*., 2001; McLaughlin *et al*., 2002). Such phenomena may in the end completely frustrate the application of broad spectrum PCR in nucleic acid extracts of these matrices.

30.3 PERSISTING PROBLEMS WITH MOLECULAR DIAGNOSTICS

In clinical microbiology, companies focus on a limited number of economically attractive microbes for the development of well-standardized, commercially available assays. From this perspective, it is deemed unlikely that the number and scope of actively marketed infectious disease tests will increase rapidly over the coming years: all of the economically interesting diagnostic areas now seem to be covered and primarily the competition between diagnostic

companies may increase significantly when different tests aiming at the same agent become more widely available. However, it should be emphasized that there is a large and still growing list of less prevalent microbial pathogens for which the commercial availability of nucleic acid test systems would be much appreciated by medical microbiologists and infectious disease specialists. For most of these minor pathogens, molecular diagnosticians have to rely on tests that have been developed in-house; a major drawback is the fact that these tests generally are not accepted and widely used. These tests usually suffer from restricted quality control only (see also Chapter 40). Hopefully, the small niche markets will raise the interest of start-up or other small companies.

Besides the current lack of specific tests availability, another bottleneck is due to the lack of convenient and reproducible nucleic acid isolation systems used in combination with the diversity of clinical specimens received by the clinical microbiology laboratory. Although several open systems are available, there still is a clear need for versatile, highly reliable automated systems. When available, these machines may significantly enhance full implementation of nucleic acid detection in the day-to-day laboratory setting. Furthermore, this will enable the laboratory to generate results within a short turnaround time, which is essential for infectious disease management. In addition, only limited amounts of information are available on the (dys)functioning of the present generation of robots and other instruments in use for diagnostic analyses. This scant availability has thus far frustrated detailed intercenter comparisons on the machines' performance. It is interesting to note that integrated platforms for both nucleic acid extraction and amplification are now available. It is particularly noteworthy that some of these machines can even be considered portable due to their small size (Seme *et al*., 2008).

Lack of standardization and quality control programmes is another persisting problem area in molecular diagnostics (see also Chapter 40). It has been known since the early 1990s that one of the most significant hurdles to be overcome by molecular technologies is false positivity due to contamination and false negativity due to the large differences in sensitivity between various test systems (Schuurman *et al*., 1996). European intercenter quality control research programs have indeed shown the clear need for standardized reference materials and the general desire to participate in quality control programs, for instance the ones organized by the Quality Control for Molecular Diagnostics (QCMD) organization (Van Vliet *et al*., 2001; van Belkum *et al*., 2007b). This latter organization, initially funded by the European Union, provides quality control schemes for an increasing number of viral and bacterial targets.

Besides the need for well-defined qualitative quality control programs, there is also a lack of standardization of (semi) quantitative assays. External standards should be developed in a format that shows maximum identity to the samples of interest. In the end this integration of molecular standards and maybe even unit definition would facilitate the intercenter communication of matching data. The conclusion still is that at present absolute quantification is very hard to achieve, a problem that may persist for years to come. Also, in the field of subspecies identification of clinical bacterial isolates, big problems have been noted. Many of the molecular technologies used for fingerprinting the genomes of microbes failed to be reproducibly implemented even in different but closely collaborating laboratories (van Belkum *et al*., 1995; van Belkum *et al*., 1998a). Although incidental successes have been reported, there is no current gold standard procedure for microbial typing (De Lencastre *et al*., 1996; Murchan *et al*., 2003). The technology most likely to survive in the end and already now providing useful tools for detailed international microbial dissemination studies probably will be multilocus sequence typing (MLST), a technology that can now be applied using Affymetrix DNA chip technology as well (Maiden *et al*., 1998; Van Leeuwen *et al*., 2003). In addition, the application of bacterial whole genome sequencing and other forms of "deep sequencing" are now becoming more accessible. These technologies will be discussed in a later section of this chapter.

30.4 MOLECULAR VIRUS DETECTION

One has to realize that the translation from experimental research tools into routine molecular diagnostics is still not completed in many cases. In virology this is mainly hampered by the fact that many of the new testing systems are really new: there are no historic alternatives for the sensitive detection procedure by non-nucleic acid methodologies, with the detection of the hepatitis virus, the human metapneumovirus, or the coronavirus causing SARS as recent examples (Su *et al*., 2002; Poon *et al*., 2003; Van den Hoogen *et al*., 2001). This renders comparative quality assessment difficult if not impossible and both laboratory scientists and clinicians need to put their trust in a test that stands largely unverified. However, the powerful versatility and reliability of nucleic acid testing has already convinced most investigators with clinical responsibilities.

The availability of unprecedented tests in virology has accelerated the development of process and quality controls (Oberste *et al*., 2000; Van Elden *et al*., 2001; Zaaijer *et al*., 1993). One of the means to measure and validate the performance of diagnostic testing is the use of external quality assessment (EQA) control programs. The QCMD network again supplies standardized reference sample panels to be used for monitoring of development and implementation of nucleic acid detection technologies, both in a qualitative and a quantitative manner (Quint *et al*., 1995; Valentine-Thon *et al*., 2001; Wallace, 2003). The use of EQA has

until recent years been focusing almost exclusively to blood-borne viruses, for which standardized tests are now commercially available. However, the need for EQA relating to the large panel of clinically relevant but commercially less interesting targets is growing (Espy *et al*., 2000; Savolainen *et al*., 2002).

Besides external quality control, virology has also excelled in the development of internal control procedures. One of these elegant control systems concerns a complete non-human seal herpes DNA virus (Van Doornum *et al*., 2003). A real-time and quantitative TaqMan assay was developed for this phocine herpes virus (PhHV). The virus can be grown relatively easily in cell culture and, hence, is available in sheer unlimited amounts. Also, a universal RNA virus was introduced as an internal control; the phocine distemper virus (PDV) could serve the same purpose as PhHV, for example for reverse transcriptase (RT) PCR applications (Niesters, 2004). The assumption here is that an intact virus, when used as universal internal control, behaves more similarly in the extraction procedure as compared to target viruses of interest, in contrast to using, for example, a plasmid as internal control. A low and fixed amount of this virus (equal to an amount giving a cut-off (Ct) value in the real-time assay of approximately 30–33 cycles of amplification) needs to be added to each clinical sample before the extraction procedure starts. In virology this mostly involves serum or plasma, although analysis of sputum and throat samples has also been successful. The internal control virus genomes are co-extracted and subsequently amplified in a quantitative manner in (currently) a separate tube.

30.5 EXAMPLES FROM BACTERIOLOGY

In all protocols, molecular diagnostic bacteriology starts with the purification of DNA or RNA from the bacteria involved. In other words, procedures aimed at the detection of a certain specific pathogen can be adapted simply to the detection of other pathogens in general. The commercially available molecular procedures suited for the detection of the sexually transmitted, intracellular pathogen *Chlamydia trachomatis* will be highlighted in the following pages. In addition, methods suited for the detection and subspecific identification of the gram-positive microorganism *Staphylococcus aureus*, often based on in-house testing, will be discussed in detail.

30.5.1 Commercial Test Systems for *Chlamydia trachomatis* Diagnosis

C. trachomatis is a microbe capable of causing two major clinical syndromes. When infecting the eye it can cause trachoma, which ultimately leads to blindness. When infecting the fallopian tubes it can cause pelvic inflammatory disease (PID), a major cause of infertility in women (for a review see Schachter, 1985). It is clear that diagnosis of *C. trachomatis* infection, in Western countries usually disseminated by sexual contacts or through vertical transmission during birth, requires some priority. Classical *C. trachomatis* diagnosis depended on the organism's capability to infect certain receptor cell-lines. Cervical swabs were taken, transported to the laboratory in a specialized conservation medium and the suspected material was inoculated into the cell culture system. The appearance of a cytopathogenic effect was indicative of the presence of *C. trachomatis* (Suchland *et al*., 2003). This test system was insensitive, laborious, expensive, and required high levels of analytical expertise. Hence, the availability of an initial DNA hybridization-based culture confirmation test spurred enthusiasm from the diagnostic community (Tenover, 1993).

In addition to the extended culture diagnosis, the first direct nucleic acid tests became available in the early 1980s of the last century. These commercial tests manufactured by GenProbe (San Diego, CA) were initially aimed at direct ribosomal RNA detection, the second generation of tests was also able to generate limited ribosomal RNA amplification (Verkooyen *et al*., 2003). Ultimately, various amplification mediated tests were developed based on technologies such as bacteriophage Qb RNA polymerase mediated amplification (Stefano *et al*., 1997), the ligase chain reaction or LCR (Blocker *et al*., 2002), and, of course, the PCR (see Verkooyen *et al*., 2003 and references therein). These tests all underwent extensive comparisons with the culture-based assay and among each other, and ultimately the PCR tests prevailed, although the LCR tests demonstrated adequate sensitivity and specificity (Pannekoek *et al*., 2003).

With the apparent preference of many researchers for the PCR, commercial developments took various quantum leaps. Roche Molecular Systems is now selling an integral PCR-based detection system called COBAS AMPLICOR. This system facilitates automated *C. trachomatis* diagnostics, not only based on the use of cervical swabs but also on less invasive, more patient-friendly urine samples (Leslie *et al*., 2003). Similarly, commercial diagnostic systems developed by Becton Dickinson and based on the Strand Displacement Amplification principle were introduced and found to be versatile and reliable as well (Bang *et al*., 2003; Cosentino *et al*., 2004). In conclusion, in a mere 15 years a drastic diagnostic change has been observed concerning the detection of *C. trachomatis*. From the cumbersome cell culture systems, to be combined with cervical swabs, we now have a new gold standard technology: the detection of *C. trachomatis* in urine can be performed with a machine generating an adequate result within half a working day. So, added to the increased sensitivity and specificity of the test system, speed and throughput also have improved significantly over the past decade (for a concise meta-analysis of some of the available data, see Table 30.1). Although detailed cost-effectiveness

TABLE 30.1 **Meta-analysis of *Chlamydia trachomatis* molecular diagnostics versus culture. Various diagnostic procedures are listed in the column on the left, some information on each and every system is indicated in the other columns. This is not a complete survey; this is meant for highlighting some of the more common technologies and their respective performance in clinical diagnostics.**

Procedure	Target sequence	Specificity	Sensitivity	Number of samples	References
Radioactive probe hybridization	Cryptic 7 kbp plasmid	91–94%	87–90%	1,214 conjunctival samples	Dean *et al.* (1989)
PCR	16S rRNA	No cross-reactivity with DNA from 13 different bacterial species	1 bacterial cell in 100,000 host cells	DNA extracts from cell lines and various bacterial species	Pollard *et al.* (1989)
PCR and radioactive RNA hybridization	Conserved plasmid	100%	100%	200 vaginal swabs	Griffais and Thibon (1989)
Probe hybridization	16S rRNA	Not discussed	10–100 picograms	Variable numbers from different sources, mainly conjunctival swabs	Cheema *et al.* (1991)
PCR-RFLP analysis	Plasmid and outer membrane protein 1 (omp1)	Not relevant	30 culture positive, 38 PCR positive	209 cervical scrapes	Lar *et al.* (1993)
LCR	Major outer membrane protein (MOMP)	All serovars positive, no cross-reaction not even with C. psittaci and C. pneumoniae	3 elementary bodies	Dilution series of serovar L2 DNA	Dille *et al.* (1993)
LCR	Plasmid and MOMP	99.8–100% (40–85% when culture efficacy was assessed)	93–98% for plasmid PCR, 68% for the MOMP PCR	1,500 urines and urethral swabs	Chernesky *et al.* (1994)
Qβ replicase	16S rRNA	85 out of 88 culture negatives were also PCR negative	1,000 molecules, five out of six culture positive ones	94 urogenital samples	Shah *et al.* (1994)
Qβ replicase and PCR	Both 16S rRNA	Both methods added three positives to the culture positives	Five elementary bodies for both methods	94 endocervical samples	An *et al.* (1995)
PCR	16S rRNA and MOMP for discordance analysis	PCR can give positive signals for two weeks after antibiotic treatment	92.7 for the PCR, 79.1% for culture after discordance analysis	1,110 cervical swabs	Goessens *et al.* (1995)
Capture PCR	C1Q capture and endogenous plasmid	Not discussed	90–95% respectively for low and high positive cultures; capture helps concentrate bacteria	71 cervical swabs including samples with high and low positivity in the culture test	Herbrink *et al.* (1995)
Transcription-mediated amplification TMA, LCR and COBAS AMPLICOR PCR	TMA: 16S rRNA LCR: plasmid Amplicor: plasmid	TMA: 98% LCR: 99% Amplicor: 99% versus culture	TMA: 90% LCR: 91% Amplicor: 96% versus culture	First void urine and urethral/cervical scrapes for 544 males and 456 females, respectively	Goessens *et al.* (1997)
TMA and COBAS AMPLICOR PCR	TMA: 16S rRNA Amplicor: plasmid	TMA: 100% Amplicor: 99%	TMA: 85% Amplicor: 97%	First void urine of 320 males and 338 females	Pasternack *et al.* (1997)

studies have not been published thus far, preliminary explorations already indicated that in populations with a prevalence of *C. trachomatis* infections over 3.9% the direct costs of PCR mediated screening programs are low, as compared to symptom-driven analyses (Paavonen *et al.*, 1998). Recently, in an evaluation of *C. trachomatis* testing with pregnant women, first-void urine specimens from 750 consecutive asymptomatic pregnant women were collected (Rours *et al.*, 2005). The COBAS AMPLICOR test was compared to the COBAS AMPLICOR test with prior DNA isolation by use of the MagNA Pure. Next, using all 750 urines, the COBAS AMPLICOR performance for individual testing was compared to pooled testing with the standard COBAS AMPLICOR procedure and subsequently to pooled testing with COBAS AMPLICOR in combination with the MagNA Pure bacterial DNA isolation kit. The sensitivity of COBAS AMPLICOR was 65% on individual and 42% on pooled urines but improved to 92% on pooled urines with the MagNA Pure bacterial DNA isolation kit, making this combination the best screening method. The *C. trachomatis* prevalence in this population appeared to be 6.4%. Additionally, the cost of the combined MagNA Pure bacterial DNA isolation kit and COBAS AMPLICOR method on pooled urines was only 56% of the cost of the standard COBAS AMPLICOR test applied to individual urines. Costs per positive case detected in the combined method were 39% of standard costs. It is noteworthy that the molecular testing for *C. trachomatis* continues to surprise the scientific community. Cases of conjunctivitis can be directly linked to maternal carriage (Rours *et al.*, 2008) whereas there are also ongoing discussions on the relatedness between premature delivery and *C. trachomatis* status (Caan, 2006).

30.5.2 Molecular Detection and Identification of *Staphylococcus aureus*

Classical *S. aureus* culture is still amenable to improvement. Novel selective growth media providing excellent yield and specificity are still strong competitors to diagnostic DNA testing. In the case of *S. aureus*, especially the detection of the methicillin-resistant version of *S. aureus* (MRSA), it is an important driving factor behind diagnostic improvement. Colonization of patients or medical personnel with this particularly antibiotic-multiresistant bug predisposes to dangerous, difficult-to-treat infections. Colonization and subsequent infection, or spread of MRSA in the hospital setting, must therefore be prevented, hence the continuous need for methods for MRSA detection and genetic identification (see Table 30.2 for a short list of currently available methods). MRSA, like methicillin-susceptible *S. aureus* (MSSA), has its prime ecological niche in the vestibulum nasi, the foremost compartment of the nose.

Diagnostic procedures are often focused on the analysis of nasal swabs. An example of a recently described, new medium is the Oxacillin Resistance Screening Agar developed by Blanc and colleagues (2003). This medium provides adequate results within 48 hours after inoculation. The most extensive comparison of culture-based assays was recently presented by Safdar and colleagues (2003), who compared 32 different media. Their conclusion was that optimal samples are taken with standard rayon swabs, the material should be enriched overnight in salt-containing trypticase soy broth, and the pre-enrichment culture should subsequently be inoculated on lipovitellin-containing mannitol salt agar containing oxacillin. Addition of an oxacillin disk on the agar surface further increased the specificity.

It has been shown that the quality of the clinical specimen is one of the prime determinants of diagnostic efficacy. For instance, nasal swabs are to be preferred over perianal or throat swabs for assessing the colonization status of a person (Singh *et al.*, 2003). However, PCR is still considered to be the future gold standard diagnostic tool, especially for the detection and identification of MRSA. Many of the *S. aureus* PCR tests in one way or another include the detection of the methicillin resistance gene encoding the penicillin binding protein PBB2′ (Miyamoto *et al.*, 2003). The first mecA-specific test was published as early as 1991 (Murakami *et al.*, 1991) and many followed over the past decade.

Testing for the presence of MRSA is clinically important. For instance, Hallin and colleagues (2003) demonstrated that PCR diagnosis was instrumental in the modification and optimization of antibiotic therapy in 7/28 (25%) of patients included in their clinical study. Also, among cardiac surgery patients the prevention of post-surgical *S. aureus* wound infections was better guided by PCR than by culture (Shrestha *et al.*, 2003). Besides PCR, other molecular amplification methodologies are also applied to the detection of *S. aureus*. A new method, isothermal signal amplification (ISA), allows for the detection of at least 2×10^5 MRSA cells (Levi *et al.*, 2003). Although the method may have its advantages, the fact that in general a time-consuming enrichment culture is required prior to ISA may ultimately be a significant obstacle. The latest development is that of real-time quantitative PCR (Borg *et al.*, 2003; Fang and Hedin, 2003; Gueudin *et al.*, 2003). Real-time procedures have been successfully applied for infection control purposes and high-throughput application has been facilitated by spectacular technological developments (Boyce *et al.*, 2008; Gilpin *et al.*, 2007; Kluytmans, 2007).

Many other nucleic acid-controlled technologies are applied in order to reach further refinement of molecular diagnostics. This includes genome sequencing and computerized or experimental comparative genomics in order to increase the number of adequate target sequences (Baba *et al.*, 2002). Recently, it was shown that whole genome sequencing could help to identify the development of mutation in the staphylococcal genome that contributes to the development of vancomycin resistance (Mwangi *et al.*,

TABLE 30.2 Examples of MRSA-specific test systems. Four categories of test systems are included. Again, this is not a complete survey; this is meant for highlighting some of the more common technologies and their respective performance in clinical diagnostics.

Test principle	Data output	References
Culture-based tests		
The **BBL crystal MRSA ID test** assesses viability by means of biochemical detection of oxygen consumption through a fluorescent compound	Fluorescence, commercially available multi-well system for assessing growth in the presence of various antibiotics	Qadri *et al.* (1994); Zambardi *et al.* (1996); Kubina *et al.* (1999)
The **soft salt mannitol agar cloxacillin test** consists of tubes containing this medium; can be used at bedside for direct swab inoculation	An indicator substance induces a color change upon the presence of MRSA	Mir *et al.* (1998)
CHROMagar Staph aureus and oxa resistance screen agar are chromogenic plate media; addition of 4 μg/ml renders the media suitable for MRSA identification	*S. aureus* colonies on the agar show a distinct purple color on Chromagar; some problems have been noted with test sensitivity	Merlino *et al.* (2000); Apfalter *et al.* (2002)
Lipovitellin salt mannitol agar is a selective medium; lipovitellin is a glycosylated, lipid binding protein present in the yolk of egg laying animals, showing homology to human apolipoprotein B	Growth or not	Verghese *et al.* (1999)
Vitek technology is developed by bioMerieux and represents automated biochemical and anti-microbial screening of bacterial isolates	Biochemical and antimicrobial profiles are produced with limited hands-on time	Knapp *et al.* (1994)
Oxacillin resistant screen agar is another chromogenic, selective culture medium	Blue stained colonies, does require follow-up identification reactions	Blanc *et al.* (2003)
Protein detection systems		
230 kilodalton *S. aureus* surface protein was found to be useful in the agglutination of non-agglutinable strains of MRSA	Experimental set-up using purified antibodies reacting with the protein	Kuusela *et al.* (1994)
MRSA screen facilitates the detection of PBP-2a in crude cell preparations	Latex agglutination system	Van Leeuwen *et al.* (1999)
Nucleic acid detection systems		
EVIGENE probe hybridization can be used for non-PCR-mediated detection of MRSA	Staining and spectrophotometry at 405 nm	Levi and Towner (2003)
Velogene rapid MRSA identification assay is a probe-mediated procedure, which uses a chimeric probe for cycling mediated recognition of MecA gene	Fluorescence value based on fluorescein, this procedure is intermediate between probe mediated and nucleic acid amplification mediated testing	Louie *et al.* (2000); Van Leeuwen *et al.* (2001); Arbique *et al.* (2001)
Nucleic acid amplification		
Multiplex PCR for nuc, Tnase and MecA facilitates detection of MRSA	Home-brew PCR, analysis of products by gel electrophoresis	Brakstadt *et al.* (1993)
Multiplex PCR for femA and mecA facilitates detection of MRSA	Home-brew PCR, analysis of products by gel electrophoresis. Has also been combined with immunoassay for product identification, including mupirocin resistance	Vannuffel *et al.* (1998); Towner *et al.* (1998); Perez-Roth *et al.* (2001); Jonas *et al.* (2002)
Combined **immunomagnetic enrichment of *S. aureus*** followed by **TaqMan PCR using the SmartCycler**	Fluorescence measurements using the SmartCycler hard- and software	Francois *et al.* (2003)
TaqMan PCR for the *S. aureus* nuc gene in combination with selective culture-based pre-enrichment	Fluorescence measurement	Fang and Hedin (2003)

2007). Such studies demonstrate that sequencing is useful for both diagnostic but also epidemiological analyses, not forgetting the linkage between genome flexibility and phenotypes. Transcription profiling using DNA chips is useful for enhancing our understanding of stage- or stimulus-specific gene expression in *S. aureus* (Mongodin *et al*., 2003). In addition, exploring the human genome for polymorphisms that can be associated with staphylococcal infection or carriage could in the end generate alternative strategies for the identification of people at elevated risk of acquiring staphylococcal infections (van Belkum *et al.*, 2007a, b; van den Akker *et al*., 2006; Wertheim *et al*., 2008).

A very important new feature of the diagnosis of *S. aureus* infection is the possibility of simultaneously detecting the presence of certain virulence genes. For instance, French studies convincingly showed that the presence of the gene for the Panton-Valentine leukocidin (PVL) confers strong disease invoking capacities upon the staphylococcus. PVL-positive strains have been implicated as causal agents in very severe cases of necrotizing pneumonia, a disease that is leading to significant mortality (Gillet *et al*., 2002). In addition, it has been shown by a Dutch research group that the presence of one or more virulence genes may lead to enhancement of impetigo in children. It was shown that the presence of the exfoliative toxin B (ETB) gene and also the PVL gene led to an increase in the number and the overall size of the impetigo lesions (Koning *et al*., 2003).

With the availability of complete inventories of putative virulence genes, as based on whole genome comparisons, the possibilities for predictive diagnosis will increase in the future: the virulence gene repertoire of a colonizing *S. aureus* strain can be assessed by molecular diagnostics, and depending on its virulence gene profile it may be decided that the strain needs to be eliminated prior to a patient undergoing surgical treatment, which reduces the risk of post-surgical wound infection.

Finally, the detection of subspecies genetic polymorphism is important for fingerprinting staphylococcal isolates and, hence, facilitates epidemiologic studies into the dissemination of clones of MSSA and MRSA. In the past, epidemiologic studies essentially were based on a strain's antimicrobial resistance profile, biochemical characteristics, or its phage type (Weiss and Nitzkin, 1971). Phage typing determines a strain's susceptibility toward infection by a large panel of lytic *S. aureus*-specific bacteriophages. Essentially, a binary code is developed consisting of alternating sensitivity or resistance toward given phages (Weller, 2000). Forty years ago, phage typing was the epidemiological gold standard and used even for successful and informative nationwide analysis of the dissemination of identical *S. aureus* phagetypes. This clonal dissemination revealed interesting staphylococcal colonization and infection dynamics and set the stage for the development of more stable and reproducible typing systems. Again, molecular microbiology provided most of the alternative possibilities. Phage typing has, for instance, been compared in detail with random amplification of polymorphic DNA, a PCR method generating DNA fingerprints consisting of non-identified DNA molecules (van Belkum *et al*., 1993). This showed that the DNA-mediated procedure was better in the sense that it appeared to be more reproducible and that its resolving power was clearly enhanced.

Over the past years a relatively large set of alternative strategies for typing *S. aureus* has become available. This includes the assessment of mutation in a variable number of tandem repeat (VNTR) loci (Sabat *et al*., 2003), a method aiming at the detection of unit number variation in certain genetic loci of fast-evolving repetitive DNA. The mechanism behind this form of DNA polymorphism is slipped strand mispairing: during replication the DNA polymerase skips or adds a repeat unit as a consequence of the complex tertiary structure of the repetitive DNA domain (van Belkum *et al*., 1998b). Multilocus analysis, so-called MLVA approaches have now been developed for all medically relevant species and for *S. aureus* multiple systems have been described (Tenover *et al*., 2007; Francois *et al*., 2005). Specific for MRSA, various methods for finetyping of the PBP-encoding gene and its neighboring sequences have been developed (Ito *et al*., 2001; Oliveira and De Lencastre, 2002); these methods usually depend on the selective PCR-mediated amplification of locus-specific sequence elements (see Fig. 30.2).

Although nearly all DNA typing methods appear to be useful for epidemiologic analysis of MRSA and MSSA (van Belkum, 2000), pulsed field gel electrophoresis (Stranden *et al*., 2003) and multilocus sequence typing (MLST; Feil *et al*., 2003) are the two methods that are currently best appreciated. Hundreds of papers have been published describing the use of PFGE for epidemiological comparisons of sets of MRSA and MSSA strains (van Belkum, 2000; see Fig. 30.2 for examples). These research efforts all strongly contributed to the current awareness of the nature of international MRSA dissemination (Aires de Sousa and De Lencastre, 2003). MLST is relatively new and defines single nucleotide polymorphisms (SNPs) in housekeeping genes. Each gene sequence is translated into an allele code and the accumulation of all (generally 7 or 8) alleles lead to an allelic profile. This can be redefined into a single digit sequence type (ST). Its resolving power is not as strong as that of PFGE but its biggest advantage is that sequencing data are extremely portable. Data can be put in a single large database and each and every individual researcher can compare his strains according to MLST type with the database entries. This facilitates worldwide comparisons on staphylococcal genotypes to be made and a number of really seminal studies on the population structure of MRSA and MSSA have recently been published (Day *et al*., 2001; Fitzgerald *et al*., 2001; Enright *et al*., 2002; Feil *et al*., 2003). Overall, five or six major MRSA clones have traveled the world and many more minor types, restricted to certain locations, have been identified.

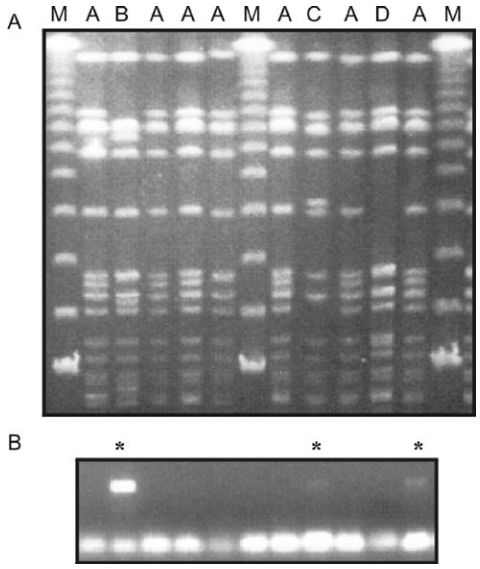

FIGURE 30.2 Gel electrophoretic analysis of genetic diversity and the presence of a methicillin resistance gene in strains of *Staphylococcus aureus*. **A.** Fingerprints generated by pulsed field gel electrophoresis (PFGE) of DNA macrorestriction fragments. Four different characteristic banding patterns are observed (indicated by the letters A to D, respectively, above each lane); these serve the purpose of unequivocal determination of relatedness between strains. Lanes marked M contain concatemeric phage lambda genomes, the smallest one being 50,000 bp in size. **B.** PCR amplification of part of the mecA gene, the gene encoding the penicillin binding protein 2 A, the product of which shows diminished affinity toward the antibiotic; hence its causal involvement in resistance. Three positive reactions are observed (marked with an asterisk).

Presently, also the allelic variation in a single staphylococcal gene can be used for epidemiological tracing of isolates within the species. The protein A-encoding *spa* genes contain a repeat region where the number of repeats is not the single variant, also the repeat sequence itself varies in sequences. So, on the basis of the number of repeats and their sequence identity, a binary code can be attributed to a strain (Strommenger *et al*., 2008). This method is highly reproducible (Aires de Sousa *et al*., 2006) and has already generated massive amounts of well-communicable data. A dedicated website exists and the *spa* typing is widely advocated as a tool that should bring national reference centers closer to their customers and, also, to their colleague centers. Even more importantly, *spa* typing is now suggested to be the first system that could possibly facilitate real-time typing in clinical settings (Mellmann *et al*., 2006). Whether this is true or false needs to be defined in well-controlled studies still.

30.6 FUTURE PERSPECTIVES

Nucleic acid-based tests are now being introduced with increasing speed into routine clinical microbiology laboratories (Check, 2001). Part of the initial and obvious delay was due to the inadequately perceived and prejudiced shortcomings of nucleic acid testing; it was supposed to be laborious, expensive, and requiring high levels of laboratory expertise (Vaneechoutte and Van Eldere, 1997). However, these historic objections are slowly being taken apart, and accelerated introduction of molecular diagnostics should be pursued in many cases. PCR testing for *Legionalla pneumophila*, the agent of Legionnaires' disease, for instance (sensitivity 80–100%, specificity more than 90%), improves significantly over culture (sensitivity 10–80%, specificity 100%) and urine antigen testing (sensitivity 70–90%, specificity 99%, Murdoch, 2003). The same is true for *Trichomonas vaginalis*, an example of a sexually transmitted parasite. Several PCR tests showing increased sensitivity and excellent specificity have been described (Van Der Schee *et al*., 1999), even using urine instead of an invasive swab as clinical specimen. Why have these tests not been implemented immediately and massively in clinical microbiology? The most frequently perceived comments in this respect still concern the supposed levels of complexity and elevated costs of the molecular tests. It is becoming clear that there is cost effectiveness in the molecular technology. In addition, it is regularly shown that clear improvement in the sensitivity and specificity of clinical testing is achieved by introducing molecular tests, so swift introduction of such tests into clinical practice will remain important to pursue.

Another important future application of molecular testing will be in the realm of host susceptibility toward infections (Relman, 2002). When genetic profiling tests for humans become more widely available (and their implications better understood) all infectious disease specialists will ultimately profit from these new molecular services. Many examples of genes important in host defense against infections have been described already (Santos *et al*., 2002; Leveque *et al*., 2003; and many, many more!!). It will be interesting to see where these exciting developments will lead over the coming decade. Whether or not this type of genetic data ultimately should be collected for each and every individual is already now a matter of intense, ethical debate. It is anticipated that for certain diseases the identification of host susceptibility factors will eventually replace microbial diagnostics.

Genomics and proteomics, not to forget metabolomics, transcriptomics, and all of the other ''omics'' sciences, have started to dominate the microbiology field over the past five years. The availability of complete genome sequences for both bacteria and the larger viruses has opened new avenues of fundamental and applied research (Cummings *et al*., 2002). From the diagnostic perspective, genomics has facilitated the characterization of novel diagnostic and epidemiologically intriguing target sequences, whereas proteomics has facilitated the identification of complete protein profiles expressed by pathogens under different environmental conditions. The use of high-throughput ''omics'' methods has enhanced our understanding of infectious diseases

significantly and the emphasis for the coming years should be on functional ''omics'' approaches, where microbiological ''omics'' data will be linked to microbial phenotypes or features of the host pathogen interaction.

30.7 CONCLUDING REMARKS

It needs to be emphasized that in principle the issues covered in this chapter for some very specific pathogens can be extrapolated to species and isolates of each and every other microbial infectious disease agent. This exemplifies the beauty of molecular diagnostics: the focused, nearly universally applicable technology is not only suited for the detection of pathogens; the subsequent genetic profiling of pathogens and assessment of their virulence potential is enabled by the same technology. Moreover, homologous technology also can be used to measure host response and eventually predict host susceptibility toward infectious diseases. It is good to witness that an obvious systems biology approach emerges upon the introduction of all of these new tools in the microbiology laboratory. The outcome of molecular diagnostic studies will continue to improve our understanding of infectious disease over the coming years. Ours are exciting times!!

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