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Current molecular techniques for the detection of microbial pathogens

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ABSTRACT

Traditionally the detection of microbial pathogens in clinical, environmental or food samples has commonly needed the prelevation of cells by culture before the application of the detection strategy. This is done to increase cell number thereby overcoming problems associated with the sensitivity of classical detection strategies. However, culture-based methods have the disadvantages of taking longer, usually are more complex and require skilled personnel as well as not being able to detect viable but non cultivable microbial species. A number of molecular methods have been developed in the last 10 to 15 years to overcome these issues and to facilitate the rapid, accurate, sensitive and cost effective identification and enumeration of microorganisms which are designed to replace and/or support classical approaches to microbial detection. Amongst these new methods, ones based on the polymerase chain reaction and nucleic acid hybridization have been shown to be particularly suitable for this purpose. This review generally summarizes some of the current and emerging nucleic acid based molecular approaches for the detection, discrimination and quantification of microbes in environmental, food and clinical samples and includes reference to the recently developing areas of microfluidics and nanotechnology ''Lab-on-a-chip''.

Keywords: *microbial pathogens, cell culture, molecular detection of microbes*

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Introduction

Microbial pathogen detection in environmental, clinical or food samples is essential for the protection of public and animal health and safety. Traditionally, methods for microbial identification require cultivation (enrichment) of the microorganisms present in a sample in selective media prior to their detection and identification. A major problem is that a large proportion of the microbial species in nature cannot be isolated or cultured using available techniques. It has been reported that the cultivability of bacteria from natural samples is much less than 1%[1]. This fact could result from a number of different issues *viz* a lack of essential nutrients in the culture medium, toxicity of the culture medium towards the organism of interest, the production of substances by other microbial species in mixed populations that may inhibit the target microorganism, the metabolic dependence of the target species on others for growth and growth stress, *etc*[2]. In any case "classical" detection methods have other limitations *i.e.* they are usually time-consuming (requiring several days or weeks to identify some bacteria), results and their interpretation often depend on the experience of the technician/scientist involved and by the mode of sample transport to the testing site and storage before testing. For all these reasons, classical methods can also be costly.

The second potential difficulty with classical methods is that they only allow a phenotypic picture of the microorganism to be constructed. This often requires that many different tests are carried out to make the picture complete. Unambiguous interpretation of results can rely on subjective judgment and personal expertise. In this context one of the major difficulties associated with microbial phenotypic identification is microbial divergence *i.e.* the fact that genotypically closely related strains of the same species can evolve significantly different phenotypes and convergence *i.e.* that genotypically distinct species can evolve similar phenotypic traits. In both cases, phenotype-based diagnostic tests can lead to misidentification.

Microscopy, usually after appropriate staining procedures, has been used to detect microbial pathogens directly *e.g.* Ziehl Neelsen (ZN) staining for Mycobacteria. However, this approach has limited sensitivity requiring a relatively large number of microbial cells even when good contrast can be achieved, as in the case of the ZN stain (when the limit of sensitivity is generally accepted to be 10^5 cells/ml sputum). Furthermore, specificity is limited because many microbial species are pleomorphic *e.g.* ZN stain cannot

distinguish between *Mycobacterium tuberculosis* and harmless environmental mycobacterial species.

Rapid immunological methods can also be used to detect microorganisms either directly or via the immune response of the host. The enzyme-linked immunosorbent assay (ELISA) is the most commonly used approach in this context. This method is low cost, can easily be standardised and is rapid. However its sensitivity is often low (about 10^4 cells can be required to produce a result), specificity can be variable and depend on the type of antibody used and dead and live microorganisms or ones from previous infections are difficult or impossible to discriminate.

For all these reasons, rapid methods based on the use of DNA, RNA and antibodies have been developed for microbial detection, identification and quantification in the last 10 to 20 years and the "ideal" approach for pathogen detection should be accurate, rapid, sensitive, reproducible, cost effective, easy to use and amenable to automation. The polymerase chain reaction (PCR) and nucleic acid hybridization-based technologies possess these characteristics.

Molecular techniques

Molecular diagnostic methods have potential advantages in sensitivity, specificity, rapidity and cost effectiveness compared to other methods for microbial identification. High specificity allows accurate identification and discrimination of microbial strains (even those with similar phenotypic traits). High sensitivity allows their use to detect microbial species or strains directly in clinical or environmental samples without the need for cultivation. Additionally, molecular methods are often more robust than conventional methods and so do not require particular care during sampling or sample transportation or a high degree of operator expertise. They may also be amenable to automation and high sample throughput.

Ribosomal RNA genes have frequently been used as target sequences for the development of nucleic acid based molecular assays because they are ubiquitous and generally present in multiple copies, have secondary and tertiary structures that are evolutionarily highly conserved but yet have primary structures composed of a mosaic of conserved and divergent sequences. These features allow rRNA to be used as one of the most phylogenetically informative markers available for microbial identification and discrimination.

Different regions of the rRNA operon have been selected as targets in various assay strategies. These include the small subunit 16S rRNA (SSU), the large subunit 23S rRNA (LSU) and intergenic spacer sequences. The choice of a particular region as a target has been based mainly on the level of variability of each region within a particular species of interest and the requirements for assay specificity and sensitivity.

In the context of rRNA target sequences, several different molecular methods, based on hybridization or amplification and sequencing of the amplified product have been developed and used to investigate microbial diversity in communities or to identify pathogens in a clinical sample. These include fluorescence *in situ* hybridization (FISH), nucleic acid fingerprinting, amplification and microarray based methods.

In the case of molecular diagnostic methods based on the detection of a particular microbial nucleic acid sequence the first step is to extract and purify the target DNA or RNA from a sample. This can represent a major difficulty. The cell lysis and nucleic acid extraction methods used on environmental, clinical and food samples are difficult to optimize as each matrix is usually different and frequently contains inhibitors detrimental to the subsequent assay. In addition, the conditions required to lyse cells with different cell wall structures vary significantly. There are very many published methods for extracting nucleic acids from environmental samples, but none of these is universally applicable. Every type of sample, because of its nature, may require a different method[3]. In this context an effective and selective extraction method (*i.e.* enrichment procedure) can be extremely useful in increasing the efficiency of molecular methods. As an example, surface modified nanometric or micrometric particles (frequently superparamagnetic iron oxide or iron oxide-silica composites) can be used in hybrid capture as effective DNA/RNA extraction tools when modified with oligonucleotides or in the immuno-magnetic separation of whole microbial cells. Effectively using such materials permits enrichment of the target of interest and removal of possible contaminating inhibitors. For a recent review on the application of magnetic particles in the development of molecular detection systems see Magnani *et al.*[4] and for other specific examples of, and applications of, such particles see refs. 5–11.

Fluorescence in situ *hybridization*

FISH permits the detection of target nucleic acid sequences (mainly rRNA) *via* the use of a fluorescently labelled probe that hybridizes to its complementary sequence within an intact cell. The oligonucleotide probes are generally small (15–25 bp in length) and covalently labelled with a fluorescent dye molecule (*e.g.* fluorescein, Cy3, Cy5, *etc.*)[12,13]. FISH is useful in detecting and determining the abundance of a particular microorganism in a given population. In FISH, samples are first fixed to a slide then permeabilized and incubated (hybridized) with the fluorescently labelled probe. After stringent washing, the labelled cells are detected by epifluorescence microscopy or flow cytometry[14]. FISH has been used for detection of a number of different microorganisms, including harmful algae in seawater samples[15], major human pathogens in clinical samples[16,17] and pathogens in food samples[18,19].

Nucleic acid fingerprinting

Whole genomes as well as specific DNA regions of microorganisms can be characterized by restriction enzyme digestion together with gel electrophoresis. The gel band patterns are referred to as DNA fingerprints and can be specific for a given microorganism or group of closely related microorganisms. Genetic fingerprinting of microbial communities can be used to determine the diversity of microorganisms living in various ecosystems and to monitor microbial community behaviour over time. Recent DNA fingerprinting techniques include denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP). In DGGE, DNA fragments amplified from a target gene (usually the 16S rRNA gene) using 'broad-range' primers are separated on the basis of sequence differences instead of size variations[20]. Polyacrylamide gels are used in this approach containing a linearly increasing gradient of DNA denaturants (a mixture of urea and formamide) (Figure 1). As the PCR product migrates in the gel, it encounters increasing concentrations of denaturants and, at some point; it starts to denature (melt). Denaturation causes a significant decrease in the electrophoretic mobility of the DNA molecule and molecules with different sequences are likely to possess different melting characteristics. Different molecules therefore migrate to different positions on the gel forming bands which can be visualized using ethidium bromide, SYBR Green I, or

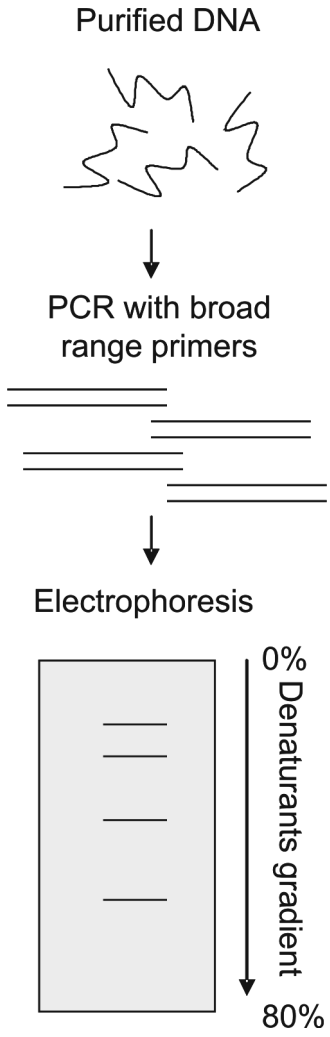

Fig. 1. *Scheme of denaturing gradient gel electrophoresis (DGGE) technique.*

silver staining. Using this method, multiple samples can be analysed at the same time and bands can be excised from gels and sequenced to allow microbial identification as well as discrimination.

T-RFLP is a modification of RFLP involving the measurement of the sizes of terminal restriction fragments from a PCR product(s). In T-RFLP, rRNA target gene sequences are PCR-amplified using one or both of the primers with a fluorescent label. The amplification product(s) are then digested with appropriate

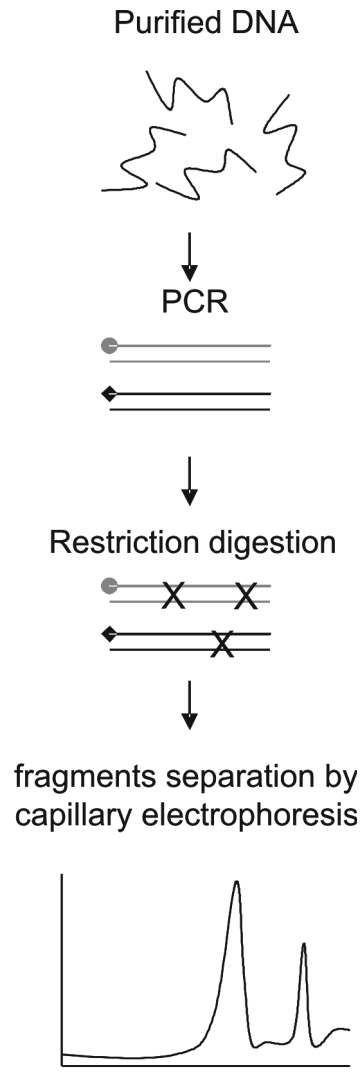

Fig. 2. *Scheme representing the terminal restriction fragment length polymorphism (T-RFLP) technique.*

restriction enzymes and resultant fragments separated by capillary electrophoresis using an automated DNA sequencer (Figure 2). The fluorescent electrophoretic profile of the digestion patterns is obtained[21]. The use of labelled primers limits the analysis (identification) to only the terminal fragments allowing the study of complex microbial communities. Moreover, the possibility of discriminating fragments with differences as small as single bases

gives the method a higher resolution than gel-based profiling techniques.

Polymerase chain reaction

One of the greatest advances in molecular diagnostic technology has probably come from the application of the PCR to the detection, discrimination and quantification of microorganisms especially microbial pathogens. PCR is extremely sensitive, potentially capable of amplifying (and therefore detecting) a single copy of a target nucleic acid sequence even in a complex mixture of molecules. Many variants of the standard PCR technique have been developed in the last 20 years but those most applied to microbial pathogen detection are: nested PCR, multiplex PCR and real-time PCR.

In nested PCR, a first 'round' of PCR amplification is used to generate a product which is itself then used as template for a second 'round' of amplification directed by a primer pair annealing internally to the target sites of the first 'round' primers. This approach can be used to increase sensitivity as a consequence of the number of amplification cycles employed, and dilute other DNA or inhibitors, and increase specificity (due to the use of a second primer set). Even if non-specific PCR products are generated in the first 'round' of amplification, these products do not generally serve as template in the second reaction[22]. The high sensitivity of the method can have a potential drawback in that it is prone to false positives particularly in circumstances where routine testing for an organism is taking place. One cause of false positives, which requires careful attention if it is to be eliminated, is the reservoir of products from previous positive reactions that may persist in the laboratory environment and which can contaminate PCR reagents (*i.e.* carry-over contamination).

In multiplex PCR, multiple primer pairs are used to amplify different target sequences in the same reaction at the same time. This allows the simultaneous detection of multiple microbial species, saving time and cost. However, when using multiple combinations of primer pairs careful attention needs to be paid to their design and testing as they need to be optimized to function under the same PCR conditions even though the primer pairs and related targets sites involved are different. In particular, primers from different pair combinations must not; form dimers, cross-anneal to each others target sites or be otherwise nonspecific (*i.e.* possess complementarity under the annealing conditions employed

with any other non-target sequence that may be present – a particular problem for samples composed of complex mixtures of unspecified microorganisms). Examples of multiplex PCR used for pathogen detection are; Kawasaki *et al*.[23] for the simultaneous detection of Salmonella spp., *Listeria monocytogenes*, and *Escherichia coli* O157:H7 in meat samples and Metherell *et al*.[24] for the simultaneous detection and discrimination of *Haemophilus parainfluenzae*, *Escherichia coli*, *Salmonella infantis*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Proteus vulgaris*, *Rhodobacter sphaeroides* and *Bacillus amyloliquifaciens*.

Conventional end-point PCR assays are usually qualitative but can be quantitative if an internal competitive standard is included in the reaction mixture. Competitive PCR is usually time consuming, complex and costly to perform. Real-time PCR on the other hand is quantitative and relatively simple to perform. In real-time PCR, a target gene is amplified with specific primers and product formation is monitored after each cycle (in real-time) by measuring a fluorescence signal. Providing the efficiency of the reaction remains constant the higher the starting quantity of the target molecule, the earlier a significant increase in fluorescence is observed. The fluorescent signal can be generated *via* several means, *viz*. by using; an intercalating fluorescent dye (*e.g*. SYBR Green I, SYBR Gold or ethidium bromide) or a number of alternative probe systems (*e.g*. TaqMan®, molecular beacons or ResonSense). The use of intercalating dyes is the simplest and least costly approach and involves adding the fluorescent dye directly to the PCR. These dyes undergo a conformational change, to become a more efficient fluorophore, on binding to double stranded DNA (dsDNA) (Figure 3A). As the concentration of dsDNA product increases with each amplification round so it is measured in real-time in the reaction tube. This makes the method a 'closed-system' and limits problems associated with carry-over contamination[25] in the testing laboratory. SYBR Green I-based assays are very sensitive but the primer's specificity for the target is crucial as any double stranded DNA is detected including any primer artefacts (*e.g*. primer dimers or non-specific target sequence independent products) which can lead to false-positive results. Also as the dye binds to all dsDNA, multiplex reactions are normally impractical. The TaqMan® approach (http://www.appliedbiosystems.com/support/apptech/#rt_pcr) depends on oligonucleotide probes complementary to a sequence located between the two primers used for PCR amplification. At one end of the probe a fluorescent reporter dye is conjugated whilst at the other terminus

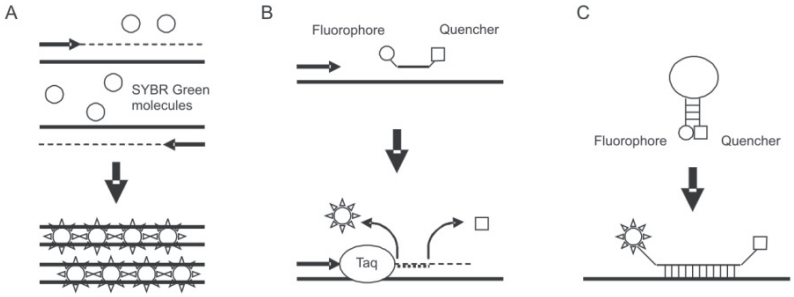

Fig. 3. *Chemistries used in real-time PCR assays. (***A**) SYBR Green is a molecule that has minimal fluorescence in the presence of single strand DNA but increases fluorescence when intercalated into double strand DNA. As PCR progresses, product formation is monitored by measuring the fluorescence signal above background at each cycle. (**B**) Taqman probes carry a fluorophore and a quencher at the 5' and 3' end, respectively, and anneal to the PCR product. The 5'-exonuclease activity of DNA polymerase cleaves the probe, releasing the fluorophore into solution allowing detection of its fluorescence. (**C**) Molecular beacons are single-stranded nucleic acid molecules with a stem-loop structure and a fluorophore and a quencher linked at each end. When the probe encounters a complementary sequence, its conformational change induces the fluorophore and the quencher to separate, generating a fluorescent signal.

there is a quencher which may be another fluor or a so-called dark-quencher (Figure 3B). In effect the structure possesses two dyes in close proximity and in this configuration the fluorescence of one (the reporter) is quenched by the other through FRET (fluorescence resonance energy transfer). During the extension step of PCR a new DNA strand is synthesised from the end of the annealed primer by the DNA polymerase. When the enzyme encounters the bound TaqMan® probe it starts to degrade the strand, close to its free 5' end, using its inherent 5'-3' exonucleolytic activity. This results in the separation of reporter from quencher and an increase in fluorescence emission can be observed if the mixture is illuminated with light at the λ_{max} excitation of the reporter molecule. The fluorescence signal is measured by the instrument in real-time at the end of the extension step in every cycle. This approach is less prone to false positive results than the intercalating dye method but is more expensive as a consequence of the need for the probe molecule. However it is possible, by the judicious choice of fluorophores, to carry out multiplex PCR using TaqMan® probes. Finally, molecular beacons are single-stranded oligonucleotide probe molecules which can adopt a stem-loop structure (Figure 3C)[26]. They are designed to be components of the PCR reaction

and to anneal to a target sequence between the two primer binding sites in the same way as a TaqMan® probe. The stem of the probe is formed by the annealing of complementary sequences at its 5' and 3' ends. The loop of the molecular beacon is the sequence complementary to the target region. A fluorophore and a quencher are covalently linked at each end of the molecule in much the same way the same way as for TaqMan®. Switching between the fluorescent and quenched states of a molecular beacon is mediated through FRET and proximity of the molecules' two functional groups. In the absence of target (*i.e.* no annealing of the molecular beacon) the fluorophore is brought into proximity with the quencher by the stem structure and no signal is emitted but when the probe loop anneals to its target sequence forming a probe-target hybrid (with greater stability than the stem structure) the fluorophore and the quencher are separated from each other and a fluorescent signal can be observed. This signal depends directly on the amount of amplified DNA present at the end of each cycle and is generally lower than the fluorescent signal generated by a TaqMan® probe which is cumulative as probe is hydrolysed in every cycle. For greater detail see http://www.molecular-beacons.org.

During real-time PCR, whichever chemistry is used, the accumulation of PCR product is measured automatically at each PCR cycle. The amount of target sequence in an unknown sample is deduced from the number of PCR cycles (threshold cycle or Ct) required to cross a fixed point above a baseline, using a standard curve as reference. External quantification standards for the construction of standard curves of Ct *versus* copy number usually consist of the target sequence cloned into a plasmid or DNA extracted from cultured cells where the concentration/copy number of the target can be determined accurately.

Real-time PCR can be used to rapidly and simply determine the quantity of target DNA sequence present even in a complex sample. The amount of DNA sequence can then be used to determine the abundance of the microbial species in a sample where the copy number of the DNA sequence (or other target) in a particular genome(s) is known.

The two main disadvantages of PCR based microbial testing are (i) the possible presence of enzymatic inhibitors in the nucleic acid template sample and (ii) the small volume of the sample that can effectively be put into a reaction. The first problem can be solved using appropriate DNA extraction methods and can be detected by measurements of PCR efficiency. The second may be overcome by concentration of target nucleic acid molecules prior

to addition to the PCR reaction *e.g.* by using a specific DNA capture probe coupled to the surface of superparamagnetic nanoparticles. Many examples of the latter approach exist in the literature relating to successful testing for viruses and microorganisms in clinical and environmental samples *e.g.* refs. 5–11.

DNA arrays

DNA flat surface microarrays consist of oligonucleotide or cDNA probes immobilized to a solid support, such as a chemically treated glass slide. Alternatively arrays can be formed from micro- or nanometric particles whose surfaces have been modified with cDNAs or oligonucleotides *e.g.* the Luminex xMAP sytem. For cDNA microarrays the surface immobilized probe is a long DNA fragment between 200 to 2000 bp in size which is typically generated by PCR and printed onto the slide using a robotic arrayer. The arrayers that are currently available commercially either use pins (see http://www.arrayit.com/Products/Printing/Stealth/stealth.html) that contact print the probe solution onto the slide surface or alternatively use inkjet technology (see http://www.chem.agilent.com/temp/rad92AF2/00033398.pdf) to deposit the solution without contact. In oligonucleotide microarrays, the probes are oligonucleotides, (usually between 20 to 70 bp in length) that can either be printed (again by pin or ink jet) or directly synthesized on the glass surface using photolithographic techniques. The first commercial microarrays produced using photolithography (http://www.affymetrix.com/technology/index.affx) depended on masks to allow photoactivation at specific locations on the microarray. A new set of masks was therefore needed for each microarray design and this requirement added a high overhead to production costs. This problem has been largely overcome by a maskless production method introduced by NimbleGen Systems Inc (see http://www.nimblegen.com/technology/manufacture.html). In the NimbleGen process the UV light needed for photoactivation is directed onto the correct locations of the microarray by projection using technology similar to that used in digital projectors. This method cuts the cost and time needed to fabricate small numbers of microarrays.

The target nucleic acid hybridized to an array can be either DNA or RNA. The first step of an array hybridization protocol is extraction of the nucleic acid from a sample of interest. Generally, unless bacteria in the sample have been enriched by

culture[27] there will be insufficient nucleic acid available in the extract for it to provide a measurable signal on the array. Consequently the next step is usually to use an amplification method such as whole genome amplification (WGA)[28], PCR or NASBA[29] to increase the quantity of target sequence present. When many target sequences must be amplified in parallel WGA[30] or a alternative such as tri-nucleotide threading[31] may represent the best option and relatively high levels of multiplexing of PCR primers has been reported[32] and random PCR primers have also been employed successfully[33]. During amplification the products become labelled with a fluor such as one of the Cy (GE Healthcare) or Alexa (Invitrogen) dyes which are available chemically linked to a nucleotide monomer. Alternatively, a non-fluorescent moiety, such as an amine or biotin can be used added to the newly synthesised amplicons. Amine groups can be linked to NHS-esters of Cy or Alexa dyes in a separate step prior to hybridization and biotin can be developed post-hybridisation to give a chemiluminescent, colorimetric or fluorescent signal using an appropriate streptavidin conjugate and substrate (*e.g.* horse radish peroxidase, alkaline phosphatase or phycoerythrin). The labelled target nucleic acids are incubated with the microarray slide under conditions promoting the annealing of complementary sequences and hybridised molecules are detected using an appropriate scanner. Fluorescence scanners are capable of very high sensitivity, resolution down to 5 μm and can measure and deconvolute fluorescent signals from multiple fluors. When more than one target (each labelled with different fluor) has been hybridized to the array, the first step is to superimpose the images produced by scanning at different wavelengths. The analysis software can then identify which pixels on two or more images refer to the same physical location on the microarray. Overlaying images can be achieved using software such as the utility provided by the ImaGene program. Figure 4 shows the result of overlaying and superimposing false colour images obtained by scanning part of a microarray of *Staphylococcus aureus* virulence genes hybridized to target DNAs labelled with different fluorophores[34]. Once superimposed probe spots and appropriate regions of background are identified then means, modes or medians can be calculated for pixels within each area (*i.e.* spots and backgrounds). The file containing this digitized microarray data can be manipulated using a wide range of computer programs from commercial *e.g.* GeneSpring (http://www.chem.agilent.com/scripts/pds.asp?lpage = 27881), BlueFuse (http://www.cambridgebluegnome.com/

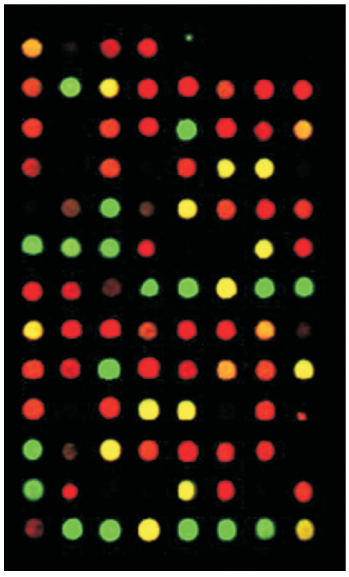

Fig. 4. *An array of oligonucleotides specific for virulence-associated gene sequences was hybridized to DNA extracted from* Staphylococcus aureus *strains Mu50 (green false colour representing Cy3) and MW2 (red colour representing Cy5). The Figure shows spots made by a single split pin. Yellow (and orange) spots show probes that hybridised to target genes in both strains. Green and red spots show probes for genes that are present in only one of the two strains. Missing spots are probes with no corresponding gene in either strain.*

bluefuse.htm), GeneSifter (http://www.genesifter.net/web/) and academic sources *e.g.* R applications from the BioConductor package[35]. Commonly, data manipulations include background subtraction, normalization, log transformation, tests of significance and graphical analyses. The advantage of microarray systems over 'traditional hybridisation' is that potentially thousands of probes can be spotted onto the microarray surface, allowing the detection of thousands of target genes simultaneously. The design and application of a microarray is depicted in Figure 5.

DNA microarrays can permit the detection of a particular species or strain within a complex community of microorganisms in an environmental, clinical or food sample[36] and/or can be used to analyze the expression of specific microbial genes in a particular sample[37]. They can also be used to enhance PCR product detection and identification, *e.g.* target sequences belonging to many different microorganisms can be amplified simultaneously in a complex sample in a single PCR reaction using 'broad-range' primers and the pool of amplicons generated can be hybridised to a microarray

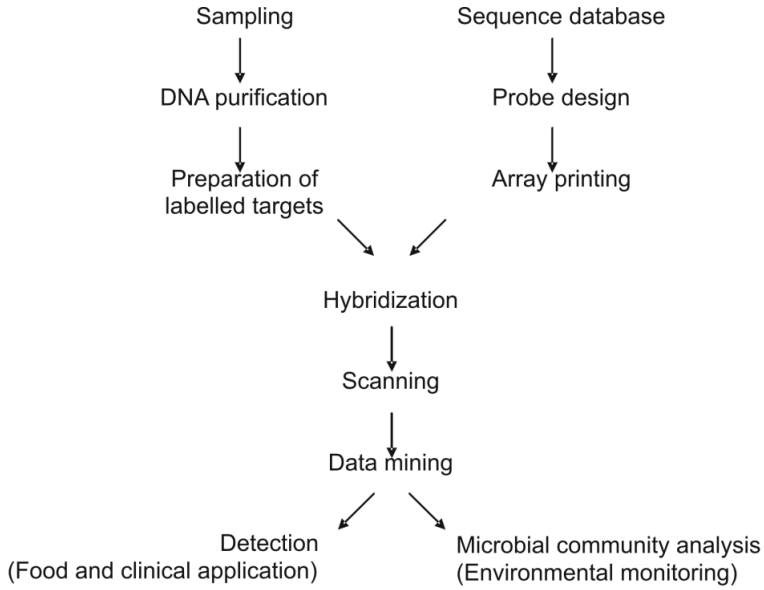

Fig. 5. *General scheme for the design and results analysis of a microarray experiment.*

carrying the species-specific probes, allowing distinction of each individual type present. It is noteworthy that, when coupled to PCR, microarrays reach sensitivity levels comparable to other molecular methods and PCR-microarrays have been used to characterise microbial communities from environmental samples[38] and to detect bacterial pathogens from a variety of sources[39].

As knowledge is increasing with respect to microbial genomics, microarrays are increasingly becoming a standard laboratory tool for both pathogen detection and their enumeration[40]. For example, using reference DNA, Cho *et al.*[41] have been able to use a microarray in estimating the target gene concentration in a sample from the hybridization signal to unhybridised array 'noise' ratio. However, the method's detection limit was approximately 10 pg, inadequate for its application to complex environmental samples.

Finally the current state of the art with respect to microarray technology means that the method suffers from high costs due to the lack of reusability and low signal to noise ratios restricting sensitivity. These factors limit the applications of arrays in routine diagnostic testing. However, the use of microarrays has been successfully demonstrated in a range of applications related to the identification and discrimination of bacteria in clinical samples

viz; *Mycobacterium tuberculosis* rifampin-resistant strains[42], to determine the pathotype of *E. coli* using oligonucleotides for virulence genes[43], to detect and discriminate between different species of *Listeria*[44], to detect pathogens in fecal samples by the use of probes targeting species-specific genes, virulence loci or 16S rRNA gene sequences[45] and to detect and characterize bacteria in samples from cases of sepsis[27].

Significant research effort has also been put into the development of an environmental multiple detection microarray aimed at identifying potential candidate pathogens involved in biological terrorism[46]. The multi-pathogen identification (MPID) microarray contains 53,660 oligonucleotides complementary to 142 unique diagnostic regions of 18 different microorganisms including bacteria, viruses and eukaryotes. Using this device, pathogen-target genes can be detected from environmental samples spiked with as little as 500 fg of pathogen DNA.

Microarrays cannot distinguish between live or viable but non cultivatable (VBNC) and dead cells since DNA can persist for long periods even after the death of cells. The ability to discriminate between live and dead cells is crucial in order to interpret the risk associated with the pathogens (especially in environmental samples). The use of a short half-life nucleic acid (*e.g.* mRNA) as the assay target may be a way to overcome this problem. Highly expressed targets could be selected in this way that would also provide enhanced sensitivity[47].

Automated and miniaturized technologies

Although molecular methods in general, and microarrays in particular, have been shown to be capable of high sample 'through-put', there has been a need to develop more automated and less expensive manual, operator based approaches in microbial testing. In fact, nucleic acid purification and the manual preparation of PCR reactions remain the most labour-intensive parts of molecular technology. New robotic instrumentation has been developed to perform these tasks and one of the first companies to launch such a system was Roche Diagnostics whose COBAS Ampliprep® and COBAS AMPLICOR Analyzer® form a semi-integrated platform. The Ampliprep automates target DNA purification using specific biotinylated oligonucleotide capture probes and streptavidin-coated magnetic beads. The second system combines thermal cycler, automatic pipettor, incubator, washer

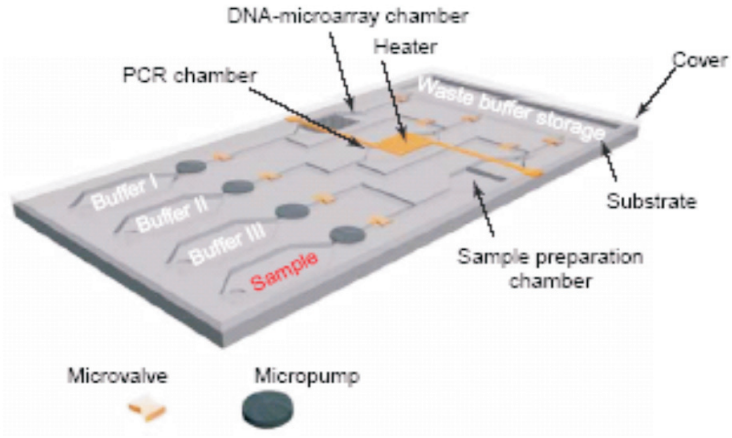

Fig. 6. *Hypothetical illustration of a laboratory-on-a-chip (LOC) device. Reproduced with permission from Liu et al.*[49].

and reader in the same instrument allowing the automated PCR amplification and detection of the target sequence.

A number of other automated systems are now available (such as the QIAGEN BioRobot®, ABI PRISM™ 6100 Nucleic Acid PrepStation® and 6700 Automated Nucleic Acid Workstation®). This equipment represents a range of purchase costs reflecting differences in processing times and sample capacities effectively making automated sample preparation and reaction automation available to all laboratories.

In environmental microbial monitoring, it could be important to carry out assays at the site of sampling and portable instruments would be required for this purpose. Rugged battery-powered versions of real-time PCR machines have been developed for use in the field. These products are aimed particularly at the detection of biothreat agents (LightCycler and SmartCycler). Kits for rapid sample preparation are available for detection of some agents. Rapid advances in miniaturization relating to nanotechnology and microfluidics have enabled the development of ''Lab-on-a-Chip'' (LOC) devices[48] which are small enough to be portable. These devices are fully-integrated, miniaturized systems which are capable of performing sample preparation together with detection in a simple and automated manner. LOCs consists of a chip containing wells, channels, electrodes and filters designed for buffer and sample storage, sample preparation, PCR and target DNA detection (Figure 6). These compartments are interconnected through microchannels to create microfluidic networks. At the microscopic

scale, physical processes such as osmotic movement and surface interactions are enhanced and reaction volumes, costs and assay time are significantly reduced, making these devices useful for field application[49]. Moreover, LOC technology allows exposure to hazardous materials to be minimized and a reduction in waste generation. Recently, Liu *et al.*[50] devised a biochip for pathogen detection consisting of microfluidic mixers, chambers, pumps, valves, channels, heaters and DNA microarray sensors. This chip was used to detect pathogenic bacteria in whole blood samples. Currently, several chip-based systems are in development or already on the market. Companies such as Agilent Technologies (Palo Alto, CA), Affymetrix (Santa Clara, CA), ACLARA Biosciences (Mountain View, CA) have products on the market based on the microfluidic LOC technology which should have significant impact on environmental microbial monitoring by permitting detection and identification of targets within minutes at the sampling site with a sensitivity level of a single cell.

Conclusions

This review has attempted to give a brief overview of current molecular techniques for the detection of microbial pathogens in food, clinical or environmental samples.

Generally, sensitive and rapid detection of microbial pathogens is now possible in a largely positive way because of the major advances which have occurred in PCR and microarray technology. Real-time PCR plays an increasingly important role in health care and environmental monitoring and has been applied for the sensitive detection of microorganisms in a broad range of environments, including water, food and animal/human tissues. Moreover, microarray technology, now allows the study of the diversity of microbial communities and to potentially test for the presence of many pathogens in a single hybridisation. However, there are some technical difficulties still to overcome such as extraction of target materials from certain samples especially complex environmental or biological ones, the availability of specific target sequences useful for the detection of specific pathogens, the resolution (signal to noise ratio) of microarray-based tests and the reusability of such approaches along with the susceptibility of PCR to contamination that can cause false positive results. However the automation and miniaturization of such assays and improved specimen-processing procedures likely to result from LOC technologies will help to overcome many of the above problems associated

with 'first-generation' molecular tests and also lead to an increase in accuracy reliability and sensitivity in testing regimes.

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