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Diagnostic Bacteriology Protocols

SECOND EDITION

Edited by

Louise O'Connor

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Diagnostic Bacteriology Protocols

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Second Edition

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Preface

The field of bacterial diagnostics has seen unprecedented advances in recent years. The increased need for accurate detection and identification of bacteria in human, animal, food, and environmental samples has fueled the development of new techniques. The field has seen extensive research aided by the information from bacterial genome sequencing projects. Although traditional methods of bacterial detection and identification remain in use in laboratories around the world, there is now a growing trend toward the use of nucleic acid-based diagnostics and alternative biochemically and immunologically based formats.

The ultimate goal of all diagnostic tests is the accurate detection, identification, or typing of microorganisms in samples of interest. Although the resulting information is of obvious use in the areas of patient management, animal health, and quality control, it is also of use in monitoring routes of infection and outlining strategies for infection control. There is, therefore, a need to ensure that the information being provided is of the highest standard and that any new technique is capable of delivering this.

Diagnostic Bacteriology Protocols, Second Edition is designed to highlight new technologies of potential use in a diagnostic setting and to outline the technological advances that have recently been made in the field of diagnostic testing. In this respect, it is hoped that *Diagnostic Bacteriology Protocols, Second Edition* will provide ideas and aid in decision making for those intending to introduce novel identification, detection, or typing technologies into their laboratories. The main considerations when implementing such new technologies include ease of use and shortened turnaround time without compromising test sensitivity or specificity. Newly developed techniques offer these advantages; in addition, they provide significant potential for multi-parameter testing and automation.

Included in *Diagnostic Bacteriology Protocols, Second Edition* are contributions by scientists at the forefront of diagnostic test development. Reviews treating current and future molecular diagnostic tests and accompanying nucleic acid extraction methods, of ultimate importance in the implementation of any molecular-based assay, are included. The protocols described in the remaining chapters range from advanced molecular detection, quantification, and typing systems to protocols for diagnostic protein identification, serological testing, and cell culture-based assays. In certain instances, the

protocols describe specific organisms that nevertheless may be easily modified for detection of other species of interest.

By including a broad range of techniques for detection of pathogens from the four main categories of bacteriology *Diagnostic Bacteriology Protocols, Second Edition* will prove of interest to microbiologists, clinicians, veterinary surgeons, and investigators involved in the field of bacterial pathogen detection and identification.

I would like to thank series editor Prof. John Walker and Mr. Thomas Lanigan of Humana Press for giving me the opportunity to become involved in this project. A sincere thanks to all contributors who have shared their expertise and knowledge in the chapters of this volume. Finally, my thanks to family, friends, and colleagues for their patience during the completion of this book.

Louise O'Connor

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A Review of Current and Future Molecular Diagnostic Tests for Use in the Microbiology Laboratory

Geert Jannes and Daniel De Vos

Summary

Nucleic acid-based diagnostics gradually are replacing or complementing culture-based, biochemical, and immunological assays in routine microbiology laboratories. Similar to conventional tests, the first-generation deoxyribonucleic acid assays determined only a single analyte. Recent improvements in detection technologies have paved the way for the development of multiparameter assays using macroarrays or microarrays, while the introduction of closed-tube real-time polymerase chain reaction systems has resulted in the development of rapid microbial diagnostics with a reduced contamination risk. The use of these new molecular technologies is not restricted to detection and identification of microbial pathogens but also can be used for genotyping, allowing one to determine antibiotic resistance or to perform microbial fingerprinting.

Key Words: Molecular diagnostics; reverse hybridization; line probe assay; microarray; real-time PCR; TaqMan®; LightCycler®; bacteria; microbial typing; fingerprinting; sequencing; mass spectrometry; broad-range PCR; antibiotic resistance; bacterial population structure; molecular epidemiology; pathotyping.

1. Introduction

The main function of all diagnostic bacteriology laboratories is the detection and identification of microorganisms in a variety of samples of human, animal, food, industrial, or environmental origin. Additionally, in clinical laboratories, drug susceptibility testing of the isolates to allow correct treatment decisions is of major importance. A third and equally important activity is epidemiological typing of the isolated and identified bacterial species. This requirement is basic for monitoring the routes of infection as well as for bacterial population studies, both essential in the setup of strategies to prevent or control infections both in the community and the heath care facility itself.

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Since the last edition of this handbook in 1995, the field of molecular diagnostics has evolved significantly. During the last decade, extensive research on microbial genomes and the development of new nucleic acid-based methodologies have resulted in the increasing use of molecular assays in the microbiology laboratory. A multitude of tests are available in different fields, mostly still as home-brew assays, but several as commercially available kits produced by major diagnostics manufacturers or by emerging new companies.

This introductory chapter provides an overview of molecular methods applied in bacterial diagnostics at the levels of detection, identification, and bacterial typing. Some important aspects of these new diagnostics in relation to conventional microbiology are discussed briefly in the context of future developments and applications for modern bacterial diagnostics.

2. Microbial Detection, Identification, and Drug-Susceptibility Testing

2.1. Phenotype-Based Methods

A first indication of the presence and nature of an organism can be obtained by direct microscopic examination of the specimen. Different staining procedures routinely are used, with the Gram stain being the most common. However, mostly the final confirmation of bacterial infections is based on culturing the pathogen in selective growth medium followed by characterization of the organism based on phenotypic criteria. The requirements for standardization, quality, efficiency, and reduced labor cost have led to the introduction of automated systems into the bacteriology laboratory for the isolation and identification of microorganisms. Continuously monitored blood culture systems such as BACTEC 9000 (BD Diagnostics, Sparks, MD) or BacT/ALERT (BioMérieux, Marcy l'Etoile, France) are standard laboratory equipment. For identification and drug-susceptibility testing, both manual and automated systems are well established. All systems are based on the miniaturization of conventional methods to reduce the volume of reagents, increase the user-friendliness, and shorten the time to obtaining a result. Manual systems such as API (BioMérieux) or Crystal ID (BD Diagnostics) require skilled users, whereas the stand-alone systems, Vitek (BioMérieux), Phoenix (BD Diagnostics), or MicroScan WalkAway (Dade Behring, Deerfield, IL), generate results automatically. Turnaround time from receipt of the sample in the laboratory to delivery of the report to the physician for culture-based methods requires minimally 24 h, but an average of 48 h or more usually are needed ***(1)***.

Rapid detection and identification of microorganisms without culture was made possible by the development of immunoassays, which are based on the specific binding between an antigen and its corresponding antibody. The assays either detect the presence of specific antibodies raised in response to an antigen or de-

tect the substance with antigenic properties itself. The technique is being used in many applications in bacteriology and is available in many different formats, such as enzyme immunoassays, immunofluorescence assays, latex agglutination assays, line immunoassays or, more recently, lateral-flow immunoassays. Rapid identification of microorganisms in clinical samples such as cerebrospinal fluid or urine by direct antigen testing is specific, easy to perform, and economical, whereas screening for specific antibodies is used mostly for serodiagnosis and allows the differentiation of acute or past infections ***(2)***. Although the different assay formats are continually being improved, antigen detection still suffers from a lack of sensitivity and, as a result of the time lag before seroconversion, serology can be ineffective for early diagnosis of infection.

2.2. Genotype-Based Methods

Both culture-based identification and immunological assays use the phenotypic characteristics of the microorganism. However, identification criteria such as colony morphology or production of certain antigens (e.g., toxins) can change or be influenced by nutritional or environmental conditions and may lead to a misinterpretation of results and subsequent misidentification of the organism. Nucleic acid-based identification methods make use of the more stable genotypic characteristics of the microorganism. Conserved regions or genes in the bacterial genome can be exploited for bacterial genus or species identification and used to define taxonomic relationships, whereas genes encoding virulence factors or toxins can be useful for defining the pathogenicity of the organism under investigation ***(3,4)***.

Direct hybridization assays using labeled oligonucleotide probes currently are used for culture confirmation or for direct detection of organisms in clinical or food samples. Probe-based assays show a high degree of specificity because, when using stringent reaction conditions, a positive hybridization signal is correlated directly with the presence of the organism. A disadvantage of direct hybridization-based assays is the need for a relatively large number of target cells. This lack of sensitivity can be partially circumvented by using ribosomal ribonucleic acid (rRNA) as a target molecule. Examples are the Accuprobe® assays (Gen-Probe Inc., San Diego, CA), which commonly are used in clinical microbiology laboratories, or GeneTrak dipstick assays (NeoGen, Lansing, MI), which mainly are applied for the identification of foodborne pathogens ***(5,6)***.

Fluorescent *in situ* hybridization (FISH) is an attractive method for the rapid detection and identification of bacteria or fungi directly from slide smears. This technology has the speed and ease-of-use of conventional staining methods combined with the specificity of molecular methods. Hybridization with fluorescent-labeled probes that target rRNA is performed on smears, and results are observed by fluorescence microscopy. An interesting new development is the

use of peptide nucleic acid (PNA) probes in FISH applications. PNA molecules have a neutral peptide-like backbone unlike the negatively charged sugar-phosphate backbone present in deoxyribonucleic acid (DNA). This unique property allows PNA probes to bind strongly and rapidly to their target sequence. This bond is resistant to nucleases and proteases, and the hydrophobic nature of the molecule increases its ability to enter the organism through the hydrophobic cell wall. PNA FISH can be used for rapid species identification of positive blood cultures and also is being applied to investigate the bacterial diversity in environmental samples ***(7)***.

The introduction of nucleic acid amplification technologies that enable the multiplication of a few target molecules to a detectable level has provided new tools for rapid, specific, and sensitive detection, identification, and resistance testing of microorganisms starting from sample material without culturing. The polymerase chain reaction (PCR) is the technique most used in research or diagnostic laboratories. The literature describes a number of home-brew PCR assays for analyses of the most important bacterial and fungal species based on species-specific DNA fragments, toxin-encoding genes, virulence factors, or broad-range primers targeting ribosomal RNA genes ***(8)***. Initially, in vitro diagnostic companies focused on the development of amplification-based assays for virological applications. Commercial assays only slowly entered the bacteriology laboratory and, at present, mainly are designed for detection and identification of slow-growing or uncultivable organisms. Alternative DNA amplification techniques such as the ligase chain reaction (LCR) have been used for bacterial diagnostics ***(9)***. For RNA amplification, techniques such as nucleic acid sequence-based amplification (NASBA) and transcription-mediated amplification (TMA) are well established in the field ***(10,11)***. Specific and sensitive detection of RNA can be advantageous, especially in food applications. PCR is based on the detection of intact DNA rather than intact viable cells and, therefore, a positive reaction may arise from either dead or live cells. Specific amplification of messenger RNA can be used for the detection of living pathogenic or spoiler organisms in food samples ***(12)***.

The analysis of the amplified nucleic acid easily can be performed by agarose gel-electrophoresis by using fragment length as an indicator for identification. The specificity of the primers used in the amplification reaction determines the accuracy of the test result. A lack of purity of the nucleic acid extract or the presence of background DNA can influence specific annealing of the primers, resulting in aspecific amplification, which can lead to misinterpretation of the results. Therefore, most of the amplification-based assays make use of hybridization probes, immobilized onto a solid support or in solution, for specific detection and identification of the amplified material.

2.2.1. Array-Based Technologies

Standard solid-phase hybridization uses labeled probes in solution to detect immobilized unlabeled amplified material or target DNA. However, most solid-phase hybridization assays are based on the reverse hybridization principle. These methods use specific hybridization probes attached to a solid support to detect labeled amplified material or target DNA in solution. Initially, in assays for detection and identification of single parameters, specific probes were immobilized in the wells of a microtiter plate ***(13)***. Later, advantage was taken of the possibility of using reverse hybridization to investigate the reactivity of an amplification product with multiple probes in a single test run. Macroarrays of fixed multiple probes at specific locations on nitrocellulose or nylon membranes were developed, with the different probes being applied as dots (dot-blots) or as lines (line probe assay [LiPA], Innogenetics, Gent, Belgium).

A LiPA strip consists of a membrane onto which synthetic oligonucleotide probes are bound as parallel lines. Precise probe design, combined with stringent hybridization and wash conditions, allows the hybridization of all oligonucleotides with the required specificity. The target region is labeled during the amplification reaction by using biotinylated primers or by incorporating biotin-labeled nucleotides. After the hybridization procedure, specific hybrids formed are visualized by an enzyme-based colorimetric procedure. Aside from applications in viral genotyping and human genetics, LiPA is an easy-to-use tool for the development of probe panels for identification of bacteria or fungi.

Given the complexity involved in detecting all relevant pathogens in a variety of biological samples, the use of a single or a limited number of target-genes for all bacteria or fungi of interest is key to the successful design of a multiparameter DNA probe-based identification and detection system. The availability of target genes that meet the requirements of broad-range amplification and specific probe design is limited. One of these target areas in the microbial genome is the spacer region between the genes coding for rRNA ***(14)***. This particular region is less conserved than the rRNA genes themselves, is present in multiple copies, and can therefore offer an excellent specificity and sensitivity for probe development. The presence of conserved rRNA sequences flanking this intervening region allows the design of a single primer set for simultaneous amplification of all bacteria or fungi present in a sample.

LiPA strips with spacer probes are used for the identification of pathogens in biological samples, as well as for culture confirmation in cases where conventional techniques are time-consuming, cumbersome, or unreliable. **Figure 1** shows the results obtained with a commercial assay, INNO-LiPA Mycobacteria v2.0 (Innogenetics), that was designed for the rapid identification of clinically relevant mycobacterial species ***(15)***.

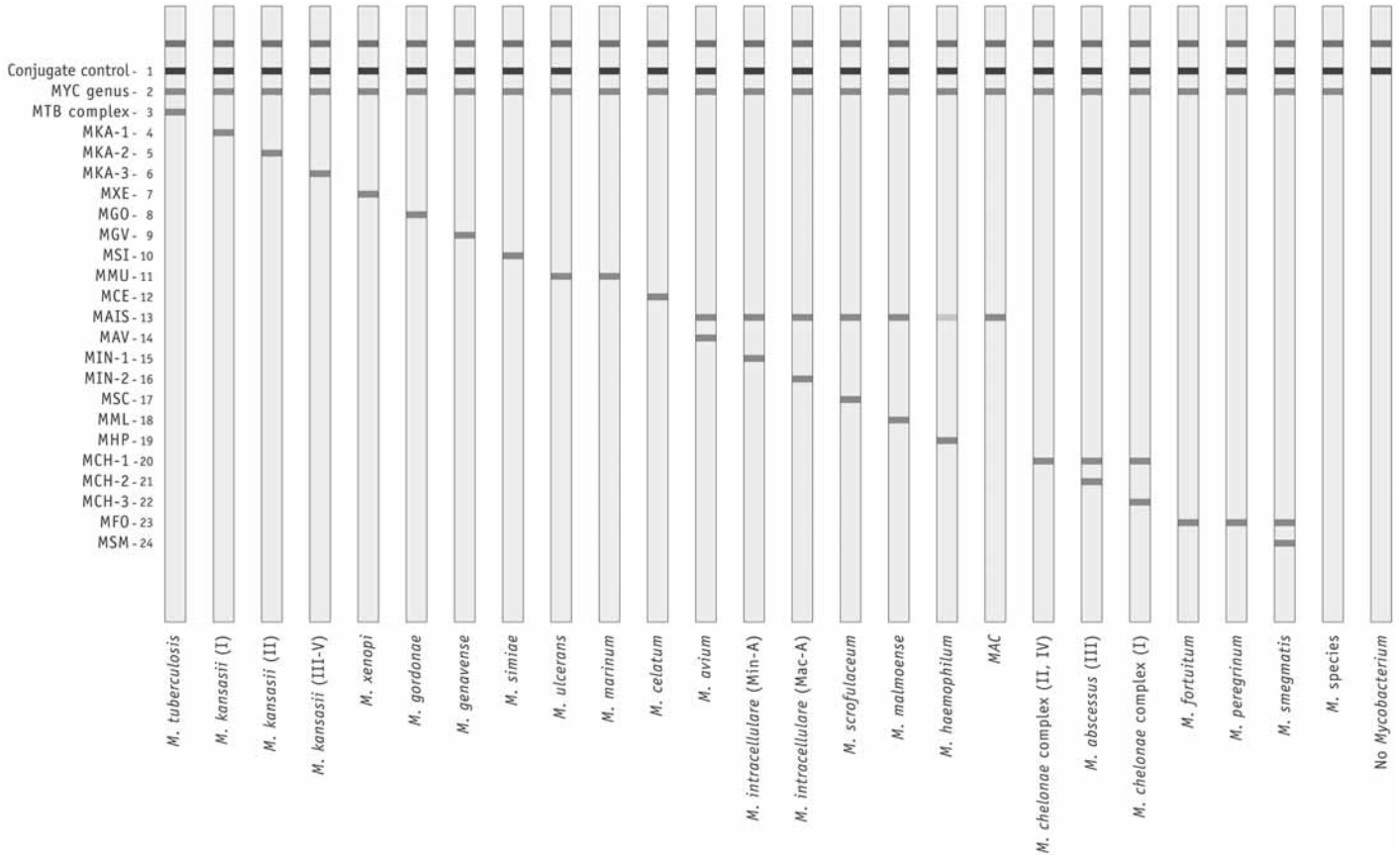

Fig. 1. Example of the different probe patterns that can be obtained with the INNO-LiPA Mycobacteria, v2.0.

Research efforts have focused more recently on the development of microarrays. As their name implies, microarrays are miniaturized versions of macroarrays, with the main difference being in the size of the probe spots. The spot sizes in microarrays are usually less than 200 to 300 µ in diameter. Specialized equipment is required for applying the probes to the solid support, and imaging systems are needed for read-outs. Whereas macroarrays are limited to fewer than 100 probes, DNA chips can vary from low-density arrays carrying a few hundred to a thousand probes, to high-density arrays containing tens of thousands of spots. The two main technologies used for the manufacture of microarrays are spotting onto the solid support using specialized dispensers and *in situ* synthesis on the array. The first approach uses pre-synthesized oligonucleotides that are deposited onto the surface of the array followed by fixation of the probes. Solid supports mostly used here are glass, silicone, or plastic slides. In the second method, the oligonucleotide probes are synthesized base-by-base directly onto the surface at predefined locations. The best-known example of this technology is the Affymetrix™ system (Affymetrix Inc, Santa Clara, CA) that uses photolithography for the production of high-density GeneChip® arrays.

The use of microarrays is well established in research projects for gene expression studies, but their use in diagnostic applications for microbiology is still in its infancy. Nevertheless, the number of publications in scientific literature describing applications in the clinical, environmental, or food-testing fields is increasing exponentially ***(16,17)***. Diagnostics companies also are developing DNA chips as illustrated by the recent introduction of an Affymetrix™ array Food-Expert-ID® (BioMérieux, Marcy l'Etoile, France) that enables the identification of the animal species composition of the sample under investigation.

High specificity, high sensitivity, a rapid turnaround, and user-friendliness are the most important requirements for microbial diagnostic assays. The PamGene 5D Pulse™ (PamGene BV, 's-Hertogenbosch, The Netherlands) microarray technology incorporates unique features that enable it to meet these objectives. The use of a three-dimensional membrane structure, which provides a 500-fold increase in reaction surface compared with a flat two-dimensional surface, allows more material to bind and enhances the probe-target reaction. A dynamic incubation using pressure variation pumps the target material through the reactive membrane maximizing the reaction kinetics. The ability to measure temperature variation and continuous monitoring of the reaction using fluorescence technology allows melting curve analysis instead of end-point detection ***(18,19)***.

Suspension arrays are based on the coupling of oligonucleotide probes to microbeads that are color-coded using different ratios of two fluorescent dyes. A third dye is used for generating labeled target DNA, which is subsequently hybridized in suspension with a set of different beads, each carrying a different

probe. The bead mixture is sorted by flow cytometry based on their internal colors and hybridized samples produce a fluorescent signal ***(20)***.

2.2.2. Real-Time Fluorescent PCR Technologies

The introduction of homogeneous or real-time PCR assays based on solution-phase hybridization methods, which combine fluorescent-labeled probes with amplification technologies, have contributed strongly to the acceptance of molecular assays in the microbiology laboratory. Several different probe technologies that are compatible with different real-time thermocycling instruments currently are available. Most of these technologies are based on the principle of fluorescence resonance energy transfer (FRET). This process occurs when two fluorophores are in close proximity to each other and the energy from an excited donor moiety is transferred to an acceptor moiety.

The so-called hydrolysis probes make use of the 5´ to 3´ exonuclease activity of *Taq* DNA polymerase. An oligonucleotide probe is labeled with a fluorescent reporter dye at the 5´ end and a fluorescent quencher dye at the 3´ end. Upon hybridization of the probe molecule to the target DNA during amplification, the 5´ nuclease activity of the enzyme cleaves the probe, separating the reporter dye from the quencher dye, resulting in a measurable increase in fluorescence. PCR thermal cycling results in an exponential amplification of the target DNA and, subsequently, of fluorescence intensity. This technology is also known as the TaqMan® system, and many applications in bacteriology are described in literature or can be purchased as commercial kits for use on real-time instruments from Roche Molecular Diagnostics (Pleasanton, CA) or Applied Biosystems (Foster City, CA ***[21,22]***).

Molecular beacons are oligonucleotide probes that emit a fluorescent signal only when hybridized to their target molecules. The oligonucleotides are able to form a stem-and-loop structure. The probe-specific sequence is located in the loop, whereas the stem is formed by two complementary sequences unrelated to the target sequence. A reporter dye is attached to the end of one arm and a quencher is attached to the end of the other arm of the stem. In closed formation, no fluorescent signal is produced, but upon hybridization, a conformational change forces the arm sequences apart and moves both dyes away from each other resulting in a fluorescent signal. Several microbiological assays using molecular beacons combined with PCR or nucleic acid sequence-based amplification have been developed for home-brew use ***(23,24)*** or as commercial diagnostics products. Examples of the latter are the IDI-Strep B™ assay, a qualitative test for the rapid detection of Group B *Streptococcus* DNA in vaginal/rectal specimens, and the IDI-MRSA™ assay, used in the direct detection of nasal colonization by methicillin-resistant *Staphylococcus aureus* (MRSA; both from GeneOhm Sciences, San Diego, CA). Both these in vitro diagnostic

assays should be performed on the SmartCycler® instrument (Cepheid, Sunnyvale, CA).

The hybridization probe format was developed for use in the LightCycler® instrument (Roche Applied Science, Penzberg, Germany) and commonly is referred to as LightCycler® chemistry (LC). Two separate oligonucleotide probes are designed to hybridize next to each other on the target DNA. The donor probe carries a 3´ label, usually 6-carboxy-fluorescein which, upon excitation, will transfer its energy to the 5´ LC dye on the acceptor probe. Any increase in fluorescence during amplification can be monitored in specific channels corresponding to the LC dye used. The recently introduced LightCycler® 2.0 instrument allows multiparameter detection using four different dyes, LC Red 610, 640, 670, and 705. Hybridization probes can be used for mutation detection based on melting curve analysis. Mismatches in the target region will cause one or both probes to dissociate at a lower temperature than the identical target sequence. Multiple amplicons or polymorphic sites can therefore be identified in the same detection channel based on different melting peaks. Numerous protocols using hybridization probes for quantitative PCR, genotyping, or mutation detection in different fields, including bacteriology, have been published ***(25–27)***.

An example of the advantage of melting curve analysis is illustrated by the LightCycler® Staphylococcus Kit M^{GRADE} (Roche Diagnostics GmbH, Mannheim, Germany). Differentiation of *S. aureus* and coagulase-negative *Staphylococcus* spp. (CoNS) is based on melting curve analysis after PCR amplification using specific primers derived from the internal transcribed spacer region. *S. aureus* will form a unique melting peak at approx 62°C, whereas CoNS give melting peaks at approx 46°C to 57°C (*see* **Fig. 2**). This manufacturer also produces similar kits designed for the detection and differentiation of *Enterococcus faecium* and *E. faecalis* or for the specific detection of *Pseudomonas aeruginosa*, *Candida albicans*, MRSA, or vancomycin-resistant enterococci. Recently, a multiparameter assay (LightCyler® SeptiFast Test; Roche Diagnostics) was launched for routine applications in critical-care medicine. This assay detects the 25 most important bacterial and fungal species causing bloodstream infections (sepsis).

Although TaqMan probes, molecular beacons, and hybridization probes are used most widely in real-time amplification, several alternative probe or primer systems have been developed, for instance, Scorpions® (DxS Ltd, Manchester, UK) or minor groove binding probes (Epoch Biosciences, Bothell, WA ***[28,29]***).

2.2.3. Sequencing-Based Technologies

Owing to rapid technological developments in equipment and reduction of cost per reaction, DNA sequencing has become established in the routine laboratory. The use of PCR-based sequencing reactions and the replacement of

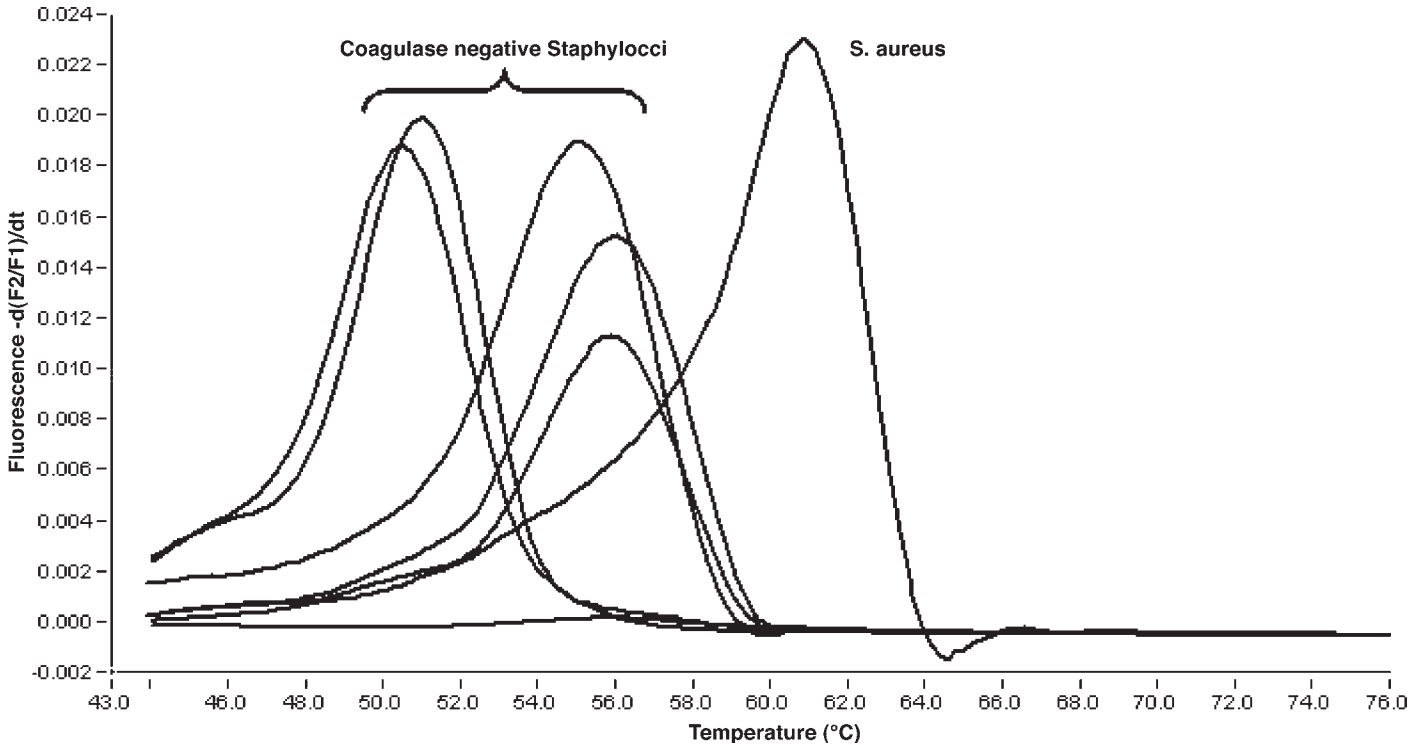

Fig. 2. Differentiation of *S. aureus* from coagulase-negative *Staphylococcus* spp. by melting curve analysis.

slab-gel for capillary-based electrophoresis instruments has made automation possible. Sequencing currently is being used for the identification of organisms that are difficult to identify using conventional methods or to detect and identify uncultivable organisms. The target studied the most for this application is the ribosomal DNA (rDNA) gene. These rDNA sequences are the cornerstone of studies on taxonomy and phylogenetic relationships between bacterial or fungal species. Thousands of ribosomal sequences are available in public as well as private databases and can be used for producing alignments and subsequent species assignment ***(30,31)***. Other conserved regions in the microbial genome, such as the ribosomal spacer region, the *rpo*B gene, the *gyr*B gene, or elongation factor Tu, can be used as alternative targets for closely related taxa ***(32,33)***.

Although Sanger-based dideoxy sequencing is the most widely used method for obtaining genetic information, other technologies such as Pyrosequencing™ (Biotage AB, Uppsala, Sweden) or mass spectrometry are promising alternatives. Pyrosequencing™ or sequencing by synthesis is applied mainly for single nucleotide polymorphism (SNP) analysis or characterization of short sequences (maximum 100 bp). It is based on an enzyme-cascade system with real-time monitoring of light produced as a result of incorporation of nucleotides. Sequence data are represented in a Pyrogram, and the peak heights reflect the number of nucleotides integrated ***(34)***. The method is accurate, easy-to-use, and data can be obtained within 1 h after target amplification. Rapid results are an advantage for diagnostic applications where time-to-result is essential, such as diagnosis of neonatal sepsis ***(35)***.

Initially, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was applied for the analysis of whole bacteria or proteins. Sequencing of bacterial nucleic acids using MALDI-TOF MS has been introduced and further improved during the last few years. Different methods to produce base-specific cleavage of nucleic acids have been described. The cleavage products are analyzed by MALDI-TOF MS, and the resulting mass patterns are compared with reference spectra for sequence determination. At this time, mass spectrometry for microbial genotyping is still in its infancy and mainly limited to academic centers or large reference laboratories. However, MALDI-TOF MS has the potential to become the method of choice for high-throughput testing. The technology gives accurate results, is fast, automatable, and cost-efficient ***(36,37)***.

3. Microbial Typing

In addition to detection and identification of the microorganism to the genus or species level, a more in-depth characterization of the isolates is an important task for the microbiology laboratory ***(38)***. Epidemiological typing not only is

important for infection control or monitoring of outbreaks but also for microbial population genetics or studies on pathogenesis. Phenotypic typing methods such as sero-, phage-, pyocin-, and antimicrobial-susceptibility typing are relatively simple and inexpensive techniques, but their discriminatory power is often of little value, especially when the organism in question undergoes physiological changes during chronic infections. Although these techniques are often a first alert for an emerging outbreak, more rapid and higher-performance systems for bacterial typing and epidemiological use are available today as the result of the advent of molecular technologies.

The most commonly used techniques for strain characterization are either based on macrorestriction analysis of total genomic DNA or make use of PCR-based methods for genome typing. Well-known examples are pulsed-field gel electrophoresis (PFGE) and ribotyping, both being established methods for bacterial subtyping in the reference laboratory. PFGE generates DNA fingerprints based on the separation of large DNA fragments after digestion of chromosomal DNA using rare-cutting restriction enzymes ***(39)***. In ribotyping, complex RFLP patterns are reduced to a limited number of fragments by hybridization with a rDNA probe. This method allows the identification of organisms to subspecies level, and the technique has been made available commercially in the RiboPrinter® Microbial Characterization System (Dupont Qualicon, Wilmington, DE ***[40]***).

Alternative techniques use PCR to amplify a number of fragments of differing length to generate DNA fingerprints. Random amplification of polymorphic DNA, or RAPD, uses short random primers at low annealing temperature to generate amplicons across the entire genome. The criticism has been expressed that RAPD lacks reproducibility between laboratories because of the use of non-stringent PCR conditions ***(41)***. This variability can be reduced by using primers directed toward specific repetitive sequences distributed throughout the genome, such as enterobacterial repetitive intergenic consensus (i.e., ERIC)-PCR, BOX-PCR, or transcription DNA-PCR.

Fingerprinting using a combination of restriction enzyme analysis and PCR is known as the amplified fragment-length polymorphism method (AFLP). This involves restriction of the genomic DNA followed by ligation of double-stranded adapters and subsequent amplification of a subset of the restriction fragments. The AFLP primers match their target sequences perfectly, that is, the adapter and restriction site sequence and a small number of selective nucleotides adjacent to the restriction sites. AFLP is a reliable and robust technique that provides an excellent performance in terms of reproducibility and resolution and has become a standard in molecular typing ***(42,43)***.

Next to these DNA fragment size-based typing techniques, DNA sequencing has become an attractive method for the characterization of isolated strains. Multilocus sequence typing is based on sequence comparison of DNA fragments derived from a set of housekeeping genes. Allelic variants are assigned for each housekeeping gene based on sequence difference and the alleles obtained for each of the investigated loci define the sequence type of the isolate. Sequence data are unambiguous and have the advantage of being submitted to databases accessible worldwide for further epidemiological studies ***(44)***. Additionally, the enormous progress made in high-throughput sequencing and bio-informatics has opened the door for whole genome sequence comparison and resulted in the new discipline of comparative genomics. The number of published microbial whole genome sequences is expected to increase exponentially, and further advancements in bio-informatics will enable scientists to compare nonvirulent and virulent strains, to study pathogenesis, and to study evolutionary relationships between microbes and their hosts. New microbial genotyping procedures that are based upon the use of sets of SNPs with predefined levels of resolution are being developed ***(45)***.

SNP-based genotyping, in combination with the detection of clinically relevant mobile genes, can result in new diagnostic assays providing both epidemiological fingerprints and information on virulence and resistance. The *Pseudomonas aeruginosa* ArrayTube (Clondiag GmbH, Jena, Germany) is such a molecular assay for strain discrimination and pathotyping. The first part of each array detects SNPs from conserved gene regions of *P. aeruginosa* allowing strain typing, whereas the second part comprises probes representing relevant pathogenicity markers and gene islands ***(46)***.

Future improvements in nanotechnology will boost the generation of comparative data on bacterial genomes. This information can provide new insight into microbial diversity as well as assist in discovering new tools to fight infectious diseases.

4. Promising Applications and Some Concerns

In view of the progress made as a result of the application of molecular biological tools in the field of microbiology in general, and in medical microbiology in particular, some aspects are being questioned and re-evaluated, although not necessarily rejected. Some of these are the “species” and “clone” concept, and “Koch’s postulates” as well as the definition of the “gold standard.” especially in specific applications like bloodstream infections and endocarditis ***(47–49)***. In the next few paragraphs, we will try to frame examples of specific applications of molecular diagnostics and some aspects of concern.

4.1. Cystic Fibrosis Associated Infections

Most patients with cystic fibrosis (CF) suffer from recurrent and chronic lung infections, which lead to deterioration of lung function, often with fatal outcome. *S.aureus* is isolated in more than 50% of the pediatric population, whereas more than 80% of adults are colonized with mucoid variants of *P. aeruginosa*. Early diagnosis of these bacteria at the colonizing stage is important for prompt adequate antibiotic treatment aimed at the eradication of the pathogen or delay in the onset of chronic infection ***(50)***. A recent study indicated that PCR-based detection of *P. aeruginosa* is useful for early detection gaining, on average, 4.5 mo over conventional culture ***(51)***.

Another comparative study between conventional and molecular methods for detection of bacteria showed that routine cultures often fail to identify bacterial species that are present in the lungs of patients with CF ***(52)***. Growth can be inhibited by antibiotic treatment, or mixed infections can be missed because of overgrowth by the most abundant organism present. Moreover, in patients with CF, the presence of auxotrophic variants of *P. aeruginosa* or small-colony variants of *S. aureus* have been described ***(53,54)***. Because these atypical variants do not grow, or grow much more slowly on routinely used media, nonculture-based molecular techniques are promising alternatives for the early detection and identification of these pathogens.

4.2. Diagnosis of Bloodstream Infections

Bloodstream infections (BSIs) are a major cause of morbidity and mortality worldwide. It has been shown that an increased time to detection of BSIs was associated with longer hospital stay and higher cost ***(55)***. The detection of bacterial or fungal growth in blood cultures currently is considered as the gold standard. Despite improvements in growth media and instrumentation, blood culture is too slow and has a poor diagnostic sensitivity and specificity. Molecular techniques for pathogen identification in positive blood cultures by FISH or PCR are able to generate faster and more specific results than conventional methods. Most attractive are amplification-based methods for direct microbial detection in whole blood, which can potentially lead to an increased diagnostic sensitivity, specificity, and shorter time-to-result and should result in a significant clinical benefit to the patient as well as savings on global hospitalization costs ***(56)***. Although first studies showed promising results, more information is needed on the clinical relevance of detecting DNA instead of living bacteria and the interpretation of positive PCR vs negative blood culture results. The use of DNA-free reagents and equipment, together with strict procedures to avoid laboratory contamination, is a critical factor for the success of this application ***(57)***.

4.3. Determination of Bacterial Load

Quantitative PCR is an established technique in the field of gene-expression studies or viral load testing. Since the successful introduction of real-time PCR instrumentation in the laboratory, the quantitative detection of bacteria for diagnostic purposes also is attracting much interest, for example, in dental health care ***(58)***. The determination of bacterial load has the potential to allow monitoring of antimicrobial therapy and it should enable discrimination among infection, colonization, or possible contamination.

In burn patients, the presence of necrotic tissue and exudates in the wound predispose to opportunistic colonization followed by enhanced bacterial invasion that often leads to fatal septicemia. The use of quantitative microbiology to monitor these patients is a major guideline in their management but often is not performed because of the high workload and time requirements of the staff. The development of a real-time quantitative PCR for detection of *P. aeruginosa* in wound biopsy samples showed that this method can provide results within 1 h with minimal hands-on-time, allowing early therapeutic decisions to be made ***(59)***.

4.4. Molecular Resistance Testing

Modern medicine is facing an increasingly important problem, namely, the threat from multidrug-resistant bacteria ***(60)***. The emergence and evolution of drug resistance is a complex and multifactorial phenomenon that requires a multidisciplinary approach if it is to be kept under control ***(61)***. This approach will be a challenge given that the phenomenon of antibiotic resistance can be viewed as a typical emergent characteristic of a dynamic, highly complex, and self-organizing system evolving at the edge of chaos ***(62)***. However, new molecular technologies offer promising tools in the fight against antibiotic resistance. Not only is rapid bacterial detection, identification, and resistance testing a significant advance, but powerful typing methods for monitoring microbial populations are also a basic requirement for containment of multidrug-resistant strains.

Currently, the main advantage of molecular resistance testing is a shorter time-to-result and improved accuracy, both of which are relevant particularly in cases of life-threatening diseases such as meningitis and sepsis, for which rapid detection, identification, and resistance testing is important, or for fastidious organisms such as *Mycobacterium tuberculosis* ***(63)***. Despite these benefits, molecular methods can only screen for known genes or polymorphisms and will not detect emerging or new resistant strains. Molecular research studies indicate that resistance is a complex system, resulting from multiple interacting mechanisms at the genomic, regulatory, and expression levels. Therefore, molecular

assays for resistance testing will not replace conventional culture-based antibiotic susceptibility testing in the immediate future, and additional technical developments in the field of multiplex amplification and DNA chips will be needed ***(64)***. Nevertheless, rapid PCR-based assays for resistance testing have been introduced in the laboratory and are excellent complementary tools, as has been shown for MRSA ***(65)***.

5. Conclusions

After the successful introduction of molecular diagnostic technologies in human genetics and virology, the number of diagnostic bacteriology protocols in medicine, food, and environmental testing has increased exponentially during the last decade. Conventional microbiology will not be replaced in the immediate future, but multiparameter identification of the most important pathogens using array-based detection technologies or closed-tube, rapid real-time PCR based assays are becoming commonplace in today's laboratories. Improvements in molecular microbial typing methods will bring epidemiological tools closer to the routine laboratory.

Further molecular research and the development of more sophisticated technologies will increase knowledge of microbial genomes and create enormous opportunities to investigate the diversity and pathogenicity of microbes and the relationship with their host. The introduction of these new technologies naturally will not only have an impact on the global organization of the microbiology laboratory but also on the training and distribution of the available resources.

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2

Overview of DNA Purification for Nucleic Acid-Based Diagnostics From Environmental and Clinical Samples

Knut Rudi and Kjetill S. Jakobsen

Summary

Direct deoxyribonucleic acid (DNA)-based detection methods are crucial for future environmental monitoring and clinical diagnosis. In this chapter, we provide an overview of of the various sample preparation approaches for bacteria for direct analyses (i.e., without culturing) in environmental and clinical samples. The issues of sampling, sample preservation, separation of the microorganisms from the environmental or clinical matrix, and DNA purification are covered. This chapter will focus on the advantages and the disadvantages of the methods available.

Key Words: NA purification; environmental/clinical analyses; direct DNA diagnostics; culture independent; polymerase chain reaction; PCR.

1. Introduction

Despite the fact that the analytical limitation in many cases is the result of the sample preparation step (e.g., separation of the cells/organisms from the environmental matrix and subsequent DNA purification), the development of new strategies in the field of sample preparation has been relatively limited ***(1)***. Microorganisms in their natural habitat may be present in low copy-numbers and in an environment that can degrade or chemically modify the nucleic acids and/or inactivate the enzymes that are used for the downstream nucleic acid analyses ***(2)***. Most sample preparation methods for DNA analyses are designed for defined materials, such as tissues and cultures ***(3)***. The challenges with natural samples are not only that the target nucleic acids are in low concentrations but also that the natural samples can be extremely heterogeneous and, in many cases, impossible to define. Finally, when analyzing nucleic acids from the environment, the issue concerning the origin of the nucleic acids is an impor-

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tant one. It is often vital to determine whether the nucleic acids are from living or dead organisms or whether contaminating organisms or nucleic acids have been introduced during processing of the samples.

The particular problems with obtaining DNA for direct analyses of microorganisms from environmental or clinical matrixes will be addressed, how these problems are currently being solved, and some possible future solutions. Also discussed will be the issues of sampling, sample preservation, separation of the microorganisms from the environmental or clinical matrix and DNA purification (*see* **Fig. 1**). The focus will be treatment after sampling because the sampling procedures will be highly dependent on the applications (environmental or clinical).

2. Sampling

Crucial sampling issues are to obtain representative samples and to keep the samples sufficiently intact for analysis in the laboratory. Normally, microorganisms are not distributed uniformly in environmental or clinical samples. Precautions have to be taken at the site of sampling to avoid modification and/or degradation of the nucleic acid in the sample. For practical reasons, the pretreatment of the sample in the field or clinic should be kept to a minimum. However, any enzymatic activity that could degrade DNA should be inactivated, in addition to the prevention of chemical inactivation and/or degradation of the DNA. The aim is to stabilize the DNA and/or microorganisms until it reaches the analytical laboratory for further treatment ***(4,5)***.

The most frequently used methods for pretreatment are either drying, freezing, preservation using alcohol, fixation in formaldehyde, or combinations of the these ***(6)***. Alcohol, such as isopropanol or ethanol, is in many cases preferable as a preservative. Alcohol is easy to use, relatively nontoxic, kills most organisms, and in it DNA is stable. Using alcohol as a preservative also may reduce the risk of accidents with clinically infectious material. Drying of the samples may be an alternative for simple sample pretreatment. The problem with drying is that the sample is not immediately preserved. DNA may be damaged or chemically modified by enzymes or chemicals while water is still present. Furthermore, microorganisms may grow during the preservation phase. However, dried samples are relatively inert and can be stored for prolonged periods ***(7)***. An approach in which the sample is squeezed onto a special paper (FTA paper) and then dried also has been applied as a successful sample preparation method ***(8)***. Snap freezing in liquid nitrogen is probably the best way to preserve a sample ***(9)***. The advantage is also is in the ability to grind the material while it still is frozen to ease the downstream DNA purification ***(9)***. However, it may not be practical to preserve the sample with liquid nitrogen freezing. Conservation with liquid nitrogen freezing requires that the sampling

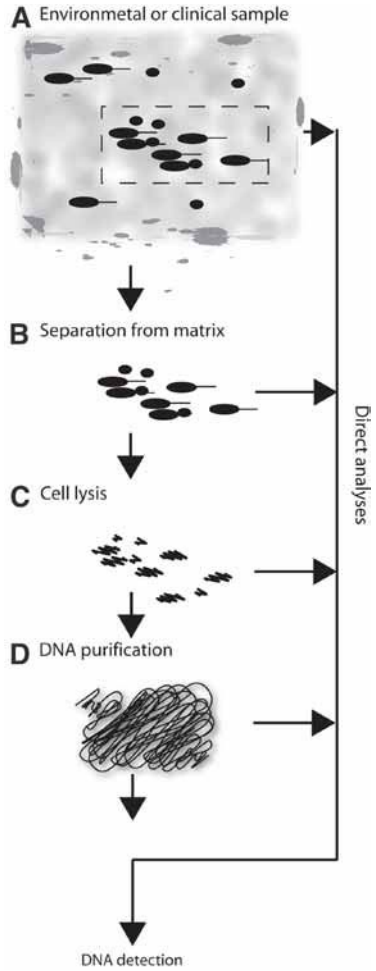

Fig. 1. Schematic representation of the process of analyzing environmental samples. The environmental sample could have a heterogeneous composition. It is important to obtain a representative sample **(A)** in the analysis of microbial communities. The bacteria are separated from the matrix **(B)** after the sampling. Then, the microorganisms are disrupted, and the DNA are released **(C)**. Finally, the DNA is purified **(D)** and is ready for downstream applications such as PCR. Steps **B** and **D** can be omitted in special cases and the DNA detected directly.

site is close to the laboratory; in addition, the sample treatment is quite extensive. When immediate preservation and stability are important issues, liquid nitrogen could be an alternative. Unfortunately, formaldehyde fixation has been a common way for sample preparation. Unbuffered acidic formaldehyde

nearly immediately destroys DNA ***(10)***, whereas buffered formaldehyde does not inactivate DNA that rapidly. However, nucleic acids are not stable over a period of time in formaldehyde ***(11)***. As opposed to formaldehyde, the iodine containing microscope fixation solution Lugol does not interfere with DNA. Lugol has been used for preservation of environmental samples that have been successfully applied for DNA analyses ***(12,13)***.

3. Separation of Bacteria From Matrix

Normally, the process of separating the cells from the environmental or clinical matrix is conducted in a laboratory. This step is important, both because major enzymatic inhibitors can be located in the matrix ***(14)*** and because of the loss of sensitivity and specificity if the DNA is isolated directly from the matrix. The sensitivity issue is of particular importance in monitoring or diagnosis of harmful or pathogenic bacteria. Microorganisms may form biofilms that are tightly attached to a surface. Critical steps are the separation of the organisms from the matrix. For soil samples, the separation of the microorganisms from the matrix can be a particular problem. The microbial cells may be tightly associated with the soil matrix, as is the case for clay particles, where the microorganisms may be bound to the particles through ionic interaction ***(15)***. Most of the methods for sample preparation from soil are thus based on direct lysis approaches ***(16)***. Recently, there has been an increased focus on microorganisms in air. This focus is both related to the possibility of biological warfare and the recognition of airborne transmission of pathogens ***(17)***. Generally, sampling from air is performed either by filtration or centrifugation. The cells are then transferred to a liquid phase before further treatment ***(18)***.

Immunocapture is a common strategy for the separation of target cells/organisms from a matrix ***(19)***. Approaches based on paramagnetic beads are the most widely applied. The paramagnetic beads are mixed with the matrix and, after complex formation between the beads and the target microorganisms, these cells can be purified through the application of a magnetic force.

Microorganisms in water and other hydrophilic liquids have been isolated and/or concentrated through unspecific adsorption onto polymer beads by lowering the water activity by the addition of alcohol and salt. This assay has been successfully applied in the analyses of cyanobacterial communities in water ***(20)***. A physical separation based on general binding properties or common affinities among whole groups of microorganisms also may be used ***(21)***. Such unspecific adsorption methods involve coating surfaces with lecithin, carbon, or metal hydroxides ***(22)***. The advantage of these strategies is that a wide range of cells can be isolated simultaneously, whereas the disadvantage is that the approach used may not be completely selective with respect to cell binding.

Copurification of undesirable compounds, or compounds that prevent the microbial binding, is a potential problem.

Generally, bacteria are relatively dense compared with most biological material and tissues. Density gradient centrifugation may thus be applied to separate the microbial cells from a biological matrix ***(14)***. This separation can be beneficial both as a result of the removal of inhibitory compounds and the fact that DNA from other organisms also may be inhibitory to polymerase chain reaction (PCR). The limitation is that the approach is quite technically challenging.

Microorganisms in liquids also can be separated by dielectrophoresis. The approach is based on inducing an uneven charge distribution within a cell by an oscillating electrical field and using this as a criterion for separation ***(23)***. This technique, however, is both sensitive to the conductivity of the medium and to particulate contaminants because of the small size of the electrophoresis unit.

Currently, no single approach for separating microorganisms from environmental or clinical matrices fulfills the requirements for diverse range of environmental matrices that exist. There are still major challenges related both to the separation of microorganisms from the environmental matrix and in the processing of large sample volumes. There has, however, been progress recently in using common physical properties among groups of bacteria to develop more general sample preparation approaches ***(21)***.

DNA analyses of complex microbial samples require a rigid lysis procedure that does not introduce errors from the differential lysis of different microorganisms in the sample ***(24)***. Mechanical, chemical, and enzymatic approaches commonly are applied. The mechanical disruption methods involve grinding of the material—either fresh, freeze dried, or frozen in liquid nitrogen. Substances such as alumina or glass beads can be added to facilitate the mechanical grinding process. The advantage of grinding is that any type of material can be processed, whereas the disadvantage is the possibility of crosscontamination and that the process can be difficult to automate. Sonication (using ultrasound) to release nucleic acids also has been successfully applied to clinical samples ***(25)***. Enzymes can be used to selectively degrade certain types of biological material, for example, for tissues mainly containing proteins, proteases can be used to degrade the matrix. Nearly all cell disruption and lysis strategies are combined with chemicals such as detergents, chaotrophic salts, and other denaturants that denature the biological material ***(26–28)***.

4. Analyses of Crude Lysates

In some special cases it is not necessary to purify the DNA from the samples. The presence of PCR inhibitors in these samples is so minimal that it will not

interfere with the PCR *(29)*, or the samples can be diluted to prevent the inhibition of enzymatic reactions *(30)*. When the amount of target material analyzed is very low, such as for the analysis of single cells or bacteria that have been concentrated by immunomagnetic separation, the DNA may actually be lost in the purification step *(31)*.

However, most environmental and clinical samples may contain compounds that are potent inhibitors of the enzymes used for analyses of DNA (**Table 1**). The inhibitors can be in the form of proteases or nucleases that degrade the polymerase or nucleic acids, respectively. Substances that destabilize the enzymes (e.g., chaotropic salts) or polysaccharides that can interact with both the nucleic acids and/or enzymes also may be potent inhibitors *(32)*. There are also compounds that may interfere directly with the polymerase activity or compounds that modify the nucleic acids *(29)*.

By adding substances that facilitate the PCR in the presence of inhibitors, or by selectively removing inhibitors from the sample, recent developments have been achieved. The advantage of such approaches is the simplicity and speed *(29)*. However, standardization of the protocols can be difficult because of the diverse nature of environmental samples.

5. DNA Purification

The classical way of purifying nucleic acids from complex-, inhibitor-, and protein-containing solutions is to apply organic solvents such as phenol/chloroform *(33)*. Other organic solvents such as chloroform or ether can be used to separate, for instance, fat from the aqueous DNA-containing phase. For algal and plant materials where co-purification of polysaccharides together with DNA may be a problem, the polysaccharides can be selectively precipitated with cetyl trimethyl ammonium bromide *(9)*. However, because of the toxicity as well as the complex handling involving centrifugation and removal of aqueous phase, DNA extractions with organic solvents are not ideal.

DNA can be bound to glass, silica particles, or other polymer surfaces in the presence of alcohol, high salt, or chaotropic agents and subsequently is released in low-salt buffers *(26)*. Other approaches using detergents *(27)* or polyethylene glycol *(28)* to bind DNA onto polymer surfaces also have been developed. The solid-phase principle has been applied in several formats, such as cartridges, filters, and paramagnetic beads. Paramagnetic beads have the advantage over other solid phases that they can easily be manipulated by a magnet and thus eliminate the need for centrifugation steps and speeding up washing steps.

The control of the yield and the purity of the isolated DNA are important parameters. The DNA quality can be measured empirically simply by evaluating the amplification efficiency of the subsequent PCR. However, such a mea-

Table 1
DNA Analysis of Bacteria in Different Matrixes

Sample type	Separation technique	Contaminants	Special Considerations
Air	Centrifugation, filtration ***(18)***	Low amount. Particles mainly.	The microorganisms are transferred to a liquid phase
Liquids	Centrifugation, filtration, binding or affinity dielectrophoresis ***(23)***	Many possible depending on liquid. However, relatively easy to define ***(20)***	Heterogeneous low amount of particles
Soil	Ion exchange, affinity binding or density gradients ***(50)***	Organic polymers, humic acids and ions ***(51)***	Heterogeneous, strong binding of microorganisms to particles
Sediments	Centrifugation ***(12)***	Similar to soil	Potential high content of dead cells
Feces	Affinity binding or density gradients ***(52)***	Proteases, nucleases, and polysaccharides ***(52)***	High content of PCR inhibitors
Plant and animal tissues	Mechanical or enzymatic disruption in combination with affinity binding or density gradients ***(33)***	Proteins, ion complexes, proteases, polysaccharides, and polyphenols	Very heterogeneous
Biofilms	Mechanical release from surface in combination with centrifugation, filtration or affinity binding	Polysaccharides	Difficult to obtain representative sample because of biofilm formation, and binding to the solid surface

surement does not give information about the kind of inhibitors present. Information about the inhibitors is crucial for the optimization of DNA extraction protocols. The main criterion for DNA purity has been measurements of protein contamination, and the most applied approach is to measure the adsorption of ultraviolet light with a wavelength of 260 nm (OD 260) and with a wavelength of 280 nm (OD 280). The OD260/OD280 ratio gives an indication of the DNA purity. For pure DNA, this ratio should be 1.7 ***(33)***. However, a ratio of 1.5 may indicate a 99% protein contamination. In addition, several pigments can interfere with the adsorption measurements ***(34)***. OD measurements do not give sufficient information for the investigation of PCR inhibitors in environmental samples. The DNA purity may be evaluated by more sensitive and specific methods to understand more about the DNA purification and the presence of potential inhibitors. Different standard methods in analytical chemistry such as matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) ***(35)***, high-pressure liquid chromatography ***(36)***, multispectral analyses, and liquid chromatography–mass spectrometry (LC–MS) yield accurate information about the different components in a sample ***(37)***. These methods, however, are not suited for routine applications but rather for optimization of the sample preparation approach.

6. Differentiation Between Viable and Dead Cells

There has been an increasing focus on the origin of the DNA purified from environmental sources. In particular, this relates to whether or not the DNA originates from viable or dead cells ***(38)***. Only approx 0.1 to 1% of the microorganisms in natural environments can be cultivated. Thus, it is not possible to determine cell viability by standard techniques ***(38)***. Soil samples, for example, often contain high amounts of free DNA in addition to DNA from dead microorganisms. Viability issues also are important in the investigations of pathogenic microorganisms in the environment, as well as in clinical settings.

DNA is, in most cases, too stable to be applied as a viable/dead marker. For instance, intact DNA has even been recovered from fossil material ***(39)***. Furthermore, the DNA stability may be dependent on both the strains and killing conditions ***(40)***. Thus, the current view is that DNA cannot be used as a viable/dead marker.

There have, however, been some recent advances in using DNA indirectly as a viable/dead marker ***(41–43)***. The principle applied is that DNA in living cells is protected by an intact cell wall/membrane, whereas these barriers are compromised in dead cells. The samples are treated with an agent that PCR inactivates the exposed DNA, resulting in a positive PCR amplification only from viable cells. There are also alternative methods being developed, such as measuring differences in the physical properties between viable and dead cells

or differences in DNA exposure ***(44)***. Separation based on physical properties can potentially be performed using the different density or dielectric properties between viable and dead cells ***(45)***. Development of methods for describing the different DNA fractions in environmental or clinical samples will be an important area for future understanding of microbial communities.

7. Future Automation

Few of the direct DNA-based methods applied for environmental or clinical analyses have been adapted for high-throughput purposes ***(46)***. For all kinds of routine diagnostic or detection purposes (usually associated with harmful or pathogenic microbes), automated protocols are likely to be the future choice. Automation of the process is a requirement for all large-scale screenings and/or to obtain reproducible results by eliminating human error.

For environmental analyses, handheld equipment that can be brought into the field is currently being developed ***(47)***. Because of the fear of biological warfare, the US army is a driving force in these developments ***(17)***. Advances also have been made in the field of pathogen control in animals used for food production ***(48)***. Future developments will be an integration of all steps into a single apparatus as in the concept of lab-on-a-chip. The current focus for lab-on-a-chip has changed from expensive silica-based to cheap plastic chips ***(49)***. These chips are gaining acceptability, mainly because they are affordable and because the liquid volumes that can be processed are in a practical range for most applications.

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3

Microarray-Based Detection of Bacteria by On-Chip PCR

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Summary

In this chapter, a protocol called on-chip polymerase chain reaction (PCR) is presented for the deoxyribonucleic acid (DNA) microarray-based detection of bacterial target sequences. On-chip PCR combines, in a single step, the conventional amplification of a target with a simultaneous, nested PCR round intended for target detection. While freely diffusible primers are deployed for amplification, the nested PCR is initiated by oligonucleotide primers bound to a solid phase. Thus, on-chip PCR allows the single-step amplification and characterization of a DNA sample as a result of separation in liquid- and solid-phase reactions. In contrast to conventional PCR, the reaction is performed directly on the flat surface of a glass slide that holds an array of covalently attached nested primers. The bacterial target DNA is amplified and probed using primers identifying either species-specific sequence regions of ribosomal DNA or unique bacterial target genes, such as virulence or resistance factors. The microarray is produced using standard spotting equipment with an array layout containing a high number of replicates. Fluorescence scanning of on-chip PCR slides allows the rapid detection of the target of interest. The protocol described herein will show how on-chip PCR can be used to detect and precisely identify DNA of bacterial origin.

Key Words: Microarray; PCR; amplification; solid-phase PCR; chip PCR; glass chip; nested primer; detection of bacteria; pathogens; ribosomal RNA; 23S rDNA; bacterial-specific genes.

1. Introduction

The rapid identification of microorganisms in clinical or environmental samples is an important challenge in modern medicine and microbiology. Molecular diagnostic-based identification of bacteria allows the accurate and sensitive analysis of DNA sequences of nearly every possible origin. During the past few years,

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DNA microarrays (which often also are called DNA or gene chips) have been extensively used for the parallel analysis of a high number of such DNA diagnostic parameters ***(1–3)***. DNA microarray protocols normally rely on the principle of nucleic acid hybridization, with hundreds to thousands of probes arrayed as spots *en miniature* onto a solid support ***(4)***. However, to detect DNA sequences and variation, a plethora of enzyme-based approaches using a DNA polymerase for nucleic acid detection has been developed. These methods often involve several separate steps, such as purification of amplification products or the preparation of single-stranded template before the actual analysis that is performed with the microarray ***(5–12)***. To overcome this, we have developed a single-step on-chip polymerase chain reaction (PCR) technique that consists of DNA sample amplification in the liquid phase and a sequence-specific nested solid-phase PCR on surface-coated glass slides ***(13–16)***. The target DNA sample is amplified directly on the flat surface of the glass chip. The chip contains several hundreds of covalently attached specific oligonucleotide primers that are suitable for interrogating multiple single nucleotide positions within the amplified sequence fragment of interest. During thermal cycling, amplification products remain covalently bound to the glass chip and can be visualized and analyzed via the incorporation of fluorescent dyes. Data interpretation is facilitated by computer-automated identification of positive reaction products, fluorescence intensity extraction, and an algorithm-based, unsupervised genotype assignment. On-chip PCR is a one-step method that provides both the sensitive PCR-based detection of nucleic acid templates and the accurate sequence information through a high number of parallel sequence and/or allele-specific solid-phase amplifications. Previously, we have successfully used the method for the detection of point mutations in human genes ***(13)***, the multiplexed and parallel analysis of single nucleotide polymorphisms ***(14,16)***, the detection of human papillomavirus in clinical samples ***(16)***, and the rapid identification of bacteria from cervical swab specimens ***(15)***. Recently, we have compared an on-chip PCR application for the detection of SNPs with state-of-the-art capillary sequencing of PCR products and demonstrated the method's validity in a routine laboratory setting ***(17)***.

In the field of clinical microbiology, the rapid identification of pathogenic microorganisms is of particular interest. In addition, the detection of mutations in drug resistance genes is important because the principal treatment of infectious illnesses is directed to reducing pathogen load by exposure to chemotherapeutic agents. Thus, for the proper treatment of bacterial infections, rapid species detection, and identification of mutations possibly conferring resistance to specific drugs would facilitate earlier effective therapy and eventually prevent the emergence of antibiotic resistance. PCR-based methods have opened new possibilities for rapid microbial detection, such that growth is no longer required for identification purposes. Many primer sets have been developed to

detect species-specific sequences in simple PCRs. However, the use of species-specific primers often is impractical in the routine analysis of clinical samples that may contain several different pathogens.

The herein-described on-chip PCR method is suited for the detection and characterization of a broad range of different bacteria in a single assay and further allows the simultaneous testing of other important diagnostic parameters, such as mutations associated with drug resistance. The method combines the amplification of either a variable region of bacterial chromosomal genes encoding ribosomal ribonucleic acid (RNA, e.g., 23S ribosomal DNA) or unique bacterial genes and the simultaneous sequence-specific detection on a solid phase. The solid phase contains species-specific, nested primers covalently bound to a glass support. During the solid-phase amplification reaction, the polymerase elongates perfectly matched primers and incorporates biotin-labeled nucleotides. The reaction products are visualized by fluorescence staining (*see* **Fig. 1**). This procedure successfully identified from pure cultures and mucosal swab samples 20 different bacterial species, in a 5-h reaction requiring very little hands-on-time. The described method can be reliably employed for the detection of any kind of sequence and/or variations in template DNA of bacterial origin and used as a microarray platform for nearly any kind of bacterial detection system.

2. Materials

2.1. Primer Design and Synthesis

1. Standard DNA oligonucleotide PCR primers.
2. Purified DNA oligonucleotides with a 5´ terminal $(CH_2)_6$-NH_2 modification (Cruachem Ltd, Glasgow, UK) for covalent slide attachment.
3. Primer3 (available from: http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) software for primer selection *(18)*.
4. GeneDoc (http://www.psc.edu/biomed/genedoc) software for *in silico* alignment of sequences *(19)*.
5. GeneRunner software for melting temperature calculations, manipulation of sequences, and the design of solid-phase primers (freely available on the internet from http://www.generunner.com/).

2.2. Coating of Glass Slides

1. Glass slides, standard microscopic 25 × 75-mm format, Melvin Brand (Sigma-Aldrich).
2. Glass trays for slides, hydrochloric acid, methanol, ethanol, dimethyl sulfoxide (DMSO; Sigma-Aldrich).
3 Trimethoxysilylpropyl-modified polyethyleneimine (Gelest). Store under argon protection once opened.

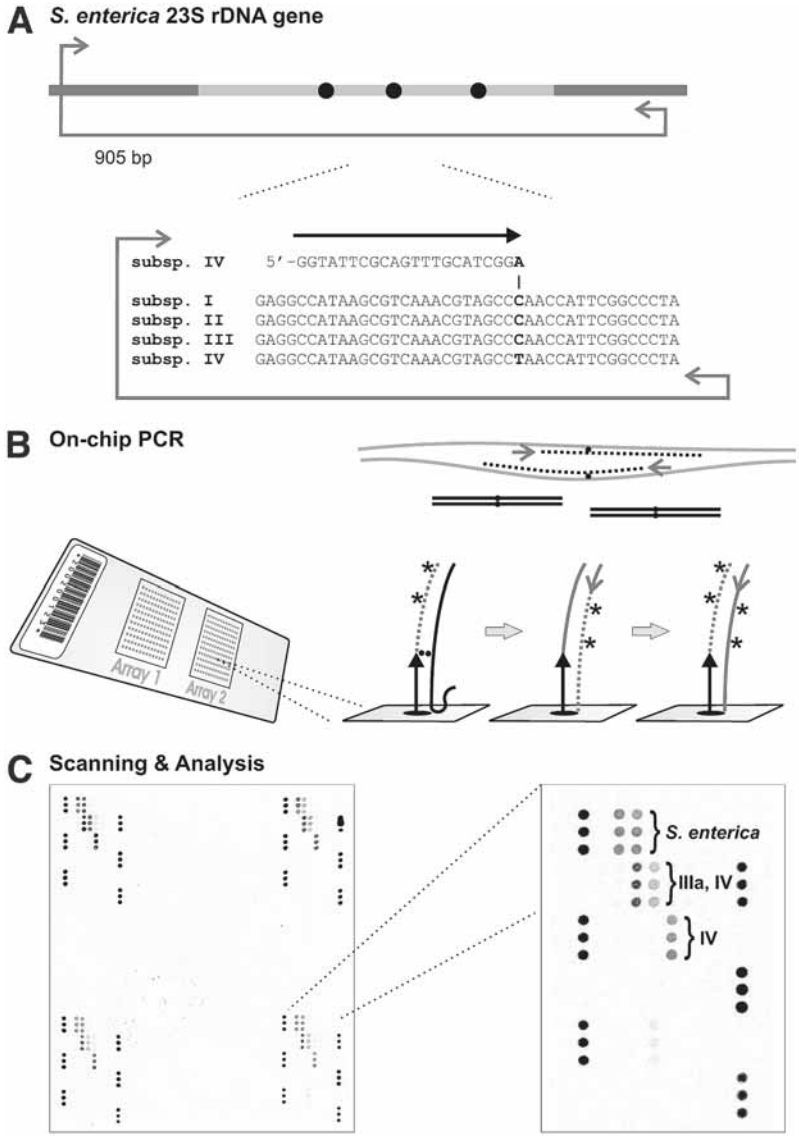

Fig. 1. Schematic overview of on-chip PCR for the detection of bacteria. **(A)** The primers are designed for interrogating variable nucleotide positions (black circles) within the *Salmonella enterica* 23S ribosomal DNA genomic region. The liquid-phase primers binding in the conserved region (dark gray) amplify a product of 905 bp that templates the (seminested) solid-phase reaction. The design rationale is shown for a solid-phase primer that allows the identification of *S. enterica* subspecies IV by probing a specific nucleotide position within the variable region (light gray). The alignment is shown in reverse orientation for clarity. *(Continued on next page.)*

4. Ethylene glycol *bis*[succinimidylsuccinate]) (EGS; Pierce). Store under argon protection once opened.
5. Plastic containers for microarray slides.
6. Vacuum sealing machine (or desiccator), desiccation bags.

2.3. Attachment of Solid-Phase Primers

1. 96-Well and/or 384-well microtiter plates.
2. Spotting buffer: 150 m*M* sodium phosphate, pH 8.5, at 24°C. Prepare fresh before use from a 2X stock solution and supplement with 0.1% (w/v) sodium dodecyl sulfate (SDS).
3. Affymetrix 417 Arrayer (Affymetrix), or similar arraying robot.
4. Plastic containers for microarray slides.
5. Vacuum sealing machine (or desiccator), desiccation bags.

2.4. On-Chip PCR

1. Blocking buffer: 150 m*M* ethanolamine, 100 m*M* Tris-HCl, pH 9.0, at 24°C. It is critical that this solution is freshly prepared on a weekly basis.
2. HotStarTaq DNA Polymerase and PCR-buffer (Qiagen).
3. dNTP set (Amersham Biosciences).
4. Biotin-16-dUTP (Biotin-16-2´-deoxy-uridine-5´-triphosphate; Roche).
5. Bovine serum albumin, PCR grade (Roche).
6. Self-Seal Reagent (MJ Research, Bio-Rad Laboratories).

Fig. 1. *(Continued)* **(B)** The glass chip contains two identical microarrays with several hundreds of covalently attached oligonucleotide primers (shown as black arrows) each. During thermal cycling, a polymerase chain reaction (PCR) product is generated in the liquid phase, serving as a template (shown as black line) for solid-phase primer extension. Elongated solid-phase immobilized products are then subject to second-strand synthesis by a liquid-phase primer (gray arrows with open heads), initiating a solid-phase PCR that is driven by the immobilized nested primer and the second primer in solution. After PCR, amplification products remain covalently bound to the glass chip and can be visualized and analyzed as the result of fluorescent-dye streptavidin conjugate staining of the biotin-labeled nucleotides (asterisks) incorporated into the amplicons during PCR. The process is shown schematically next to the slide for an example spot with a perfect match primer that allows extension at the targeted nucleotide of interest. **(C)** Detection by fluorescence scanning. As reaction products remain covalently bound to the glass surface throughout the on-chip PCR, they can be detected *via* standard fluorescence scanning of the glass chip. Subspecies-specific genotype information is deduced from reading the fluorescence intensities. The brackets indicate primer spots with positive reaction products, leading to the detection of *S. enterica* subspecies IV. Guide dots on the left and right margins serve as staining controls and facilitate orientation. The application shown was developed to detect food-borne *Salmonella*, and to discriminate between known *S. enterica* subspecies.

7. Glass cover slips (22 × 22 mm), for microarray applications.
8. PTC 200 *in situ* slidecycler (MJ Research [Bio-Rad]).
9. Phosphate-buffered saline (PBS) buffer: 0.14 *M* NaCl, 2.7 m*M* KCl, 10 m*M* Na_2HPO_4, 1.8 m*M* KH_2PO_4, pH 7.4 (as required during DNA extraction).

2.5. Fluorescence Staining and Scanning

1. Standard saline citrate buffer: 150 m*M* sodium chloride, 15 m*M* sodium citrate, pH 7.0. Store as 20X stock solution.
2. 10% (w/v) SDS solution.
3. Streptavidin-Alexa Fluor 647 conjugate (Molecular Probes). Store under light protection according to the manufacturer's instructions.
4. TBST buffer: 150 m*M* NaCl, 10 m*M* Tris-HCl, 0.5% Tween-20, pH 8.0.
5. Affymetrix 428 laser scanner (Affymetrix), or similar microarray scanner.
6. GenePix software (Axon Instruments), or any other software for microarray analysis.

3. Methods

The methods described in this heading outline the primer design rationale, the coating of glass slides, the fabrication of microarrays, the on-chip PCR, and the final processing steps of the slides and data analysis.

3.1. Primer Design and Synthesis

As an example, we describe the primer design procedure for a sequence detection system based on the use of universal liquid-phase primers (broad range PCR primers) for the amplification of 23S ribosomal DNA (rDNA) sequences. However, many other bacterial specific sequences (e.g., specific host range factor genes or virulence genes) may be used as targets for the herein described on-chip PCR method for the detection of bacterial sequences. Additionally, the liquid-phase PCR can be performed with multiple primers simultaneously amplifying universally distributed sequences and bacterial-specific sequences in a single, multiplexed reaction (*see* **Note 1**).

3.1.1. Design of Universal Liquid-Phase Primers

1. The universal primer pair targets conserved regions present in all bacteria to be examined on the panel to be designed. Furthermore, the amplified fragments must contain variable regions that provide a basis for species discrimination.
2. Primer binding sequences are located in conserved regions within helix 43 and helix 69 of bacterial 23S rDNA, present in all bacteria. The sequence region between helix 43 and 69 shows a high degree of variability between different bacterial species.
3. The PCR primers for the liquid phase are designed as oligonucleotides 20 to 24 nucleotides in length and annealing temperatures of 64 to 66°C, using the web-based software *Primer 3* (*see* **Note 2**).

3.1.2. Design of Species-Specific Solid-Phase Primers

1. For the development of bacterial-specific primers, all partial 23S rDNA sequences of the bacterial identification panel are aligned using the multiple sequence alignment computer program GeneDoc.
2. The sequences are obtained either by prior sequencing or from public databases (e.g., the EMBL nucleotide sequence database, available from: http://www.ebi.ac.uk/embl/).
3. The alignment is screened for nucleotide positions unique to specific species.
4. The solid-phase primers are designed such that the 3´ end interrogates the species-specific nucleotide positions. Include positive and negative control primers here. Positive controls interrogate conserved nucleotide positions and allow control of the successful amplification with the universal primer set. Negative controls can be used for monitoring specificity.
5. The specificity of the primers is then tested against known bacterial sequences using the *blastn* algorithm (Available at http://www.ncbi.nlm.nih.gov/blast/).
6. The primers are designed with a length between 30 and 40 nucleotides and annealing temperatures between 68 and 72°C.
7. The primers are synthesized with a 5´ terminal $(CH_2)_6$-NH_2 modification (*see* **Note 3**).

3.2. Coating of Glass Slides

3.2.1. Cleaning

1. The glass slides are cleaned by incubation in a solution containing HCl:methanol (1:1) for 24 h at room temperature (*see* **Note 4**).
2. The slides are then washed thoroughly with deionized water and dried with compressed air.

3.2.2. Silane Derivatization

1. Incubate the cleaned slides in a solution containing 3% (v/v) trimethoxysilylpropyl modified polyethyleneimine (*see* **Note 5**) in 95% ethanol for 5 min with vigorous agitation using a magnetic stirring bar at room temperature. It is important that the silane is freshly diluted from a stock stored under argon protection.
2. Wash the slides in 95% ethanol to remove remaining silane and dry using compressed air.
3. Cure the silane layer by baking the slides in an oven at 80°C for 1 h.

3.2.3. Surface Activation

1. Treat the slides with EGS by pipetting 100 µL of a 100 m*M* solution in DMSO between two slides. It is important that the EGS is freshly prepared from a stock stored under argon protection.
2. Place the two-slide sandwich upon a strip of parafilm on the laboratory bench and incubate overnight at room temperature.
3. Wash the slides in glass trays containing 250 mL of water, dry using compressed air, and seal in plastic containers using either vacuum sealing or desiccation bags.

3.3. Attachment of Solid-Phase Primers

3.3.1. Dissolving Oligonucleotides for Spotting

1. Dissolve the primers in spotting buffer at a concentration of 20 µ*M* (*see* **Note 6**).
2. Pipet 20 µL of DNA primer solution into either 96-well or 384-well microtiter plates depending on the spotting robot used.

3.3.2. Spotting of Glass Slides

1. Spot the slides using an Affymetrix 417 Arrayer equipped with 125 µm pins, which results in spots approx 200 µm diameter.
2. Before and during spotting, ambient temperature and air humidity must be controlled and maintained at 20°C and 50 to 60% relative humidity, respectively (*see* **Note 7**).
3. The spotting layout (*see* **Note 8**) is determined by the print head geometry and the well positions of specific DNA oligos within the plates. Each slide can easily hold two arrays, which require a 22 × 22-mm area for on-chip PCR.

3.3.3. Binding to Solid Support

1. Transfer the arrayed slides from the arraying robot into a humid chamber (NaCl-saturated) and incubate at room temperature for 16 h.
2. Transfer the glass slides directly into plastic containers and store at room temperature, either vacuum-sealed or using desiccation bags (*see* **Note 9**).

3.4. On-Chip PCR

3.4.1. Preparation of PCR Master Six

1. Prepare a master mix as follows. A 13-µL final volume for each reaction is used.

2X	HotStarTaq PCR buffer
100 µ*M*	of dATP, dGTP, dCTP, and 65 µ*M* dTTP
35 µ*M*	Biotin-16-dUTP
0.25 µg/µL	bovine serum albumin
1 U	HotStarTaq DNA polymerase
1.4 µ*M*	of each liquid-phase primer (*see* **Note 10**).

3.4.2. Blocking of Glass Slides

1. Immediately before use in on-chip PCR, immerse the glass slides in blocking buffer for 20 min at 55°C. This blocking step is very important because it neutralizes any residual un-reacted amine-reactive EGS groups.
2. Wash the slides with deionized water and dry using compressed air.
3. Put the slide on a clean and flat surface, ideally above a printout template, which visually indicates the positions and areas covering the two oligonucleotide arrays.

3.4.3. Starting the Reaction

1. Add the required amount of Self-Seal Reagent (25% v/v) to the prepared master mix from **Subheading 3.4.1.** and mix.

2. Add a 13-µL aliquot to separate tubes containing the DNA template (*see* **Note 11**) and mix carefully using the micropipet.
3. Pipet the master mix onto the slide, directly down onto the oligonucleotide array.
4. Immediately seal the reaction droplet by mounting a cover slip (*see* **Note 12**) using plastic forceps. Take care not to trap air bubbles when sealing the reaction droplet underneath the cover slip. Always use gloves when handling slides and do not touch the slide on the upper, flat surface containing the spots.

3.4.4. Thermal Cycling

1. Transfer the glass slides into a PTC 200 slide thermocycler and carry out the PCR according to the following scheme:

   80°C for 10 min, 95°C for 5 min
   10 cycles at 95°C for 30 s, 66°C for 25 s, and 72° C for 35 s, followed by
   25 cycles at 95°C for 20 s, 66°C for 10 s, and 72°C for 20 s, with a final extension step of 3 min at 72°C.

2. The cycling parameters could require adjustment and empirical optimization, depending on target sequence context and length.

3.5. Fluorescence Staining and Scanning

3.5.1. Fluorescence Dye Staining

1. After cycling place the slides in a glass tray filled with washing buffer containing 0.1X standard saline citrate supplemented with 0.1% SDS and incubate with gentle agitation until the cover slips swim off (takes approx 10 min).
2. Wash the slides again in fresh buffer as directed previously for 10 min, followed by a short rinse in deionized water (*see* **Note 13**).
3. Dry the slides using compressed air.
4. Stain the slides by pipetting a 20-µL droplet of staining solution containing 0.02 µg of Streptavidin-Alexa Fluor 647 conjugate in TBST buffer onto the array (*see* **Note 14**).
5. After a 2-min incubation at room temperature, wash the glass slides for 5 min in TBST buffer to remove excess dye. Rinse with deionized water and dry under an air stream.

3.5.2. Fluorescence Scanning

1. Scan the slides at 10-µm pixel resolution with excitation at a wavelength of 635 nm using an Affymetrix 428 laser scanner according to the manufacturer's instructions.
2. The scanner yields a 16-bit gray scale image that is saved for further data analysis.
3. Using the GenePix software, the fluorescence scan images are analyzed by placing a grid onto the image and performing the required spot finding and fluorescence intensity extraction steps.
4. After checking for inter-replicate consistency between the subarrays, a mean fluorescence value is calculated for each specific probe.

5. This allows assignment of final results according to the spotting layout (*see* **Note 15**).

4. Notes

1. To avoid multiple liquid-phase primers, it is more straightforward to use a single universal primer pair for the simultaneous amplification of conserved stretches of DNA from any bacterium that possibly is present in the sample. Universal primers that recognize conserved sequences of bacterial chromosomal genes encoding ribosomal RNA are particularly useful because the resulting amplified rDNA sequences also contain variable regions and provide a reliable basis for the analysis of phylogenetic relationships among different bacteria. Previous investigators usually have chosen 16S rDNA as a target for universal primers. However, the 23S rDNA region has been suggested to be more useful for clinical diagnosis because of a higher degree of variation between bacterial species of medical importance ***(20,21)***. The website of The European Ribosomal RNA database provides a comprehensive list of conserved primer sequences for the amplification and sequencing of variable regions in bacterial 23S rDNA (available at http://www.psb.ugent.be/rRNA/primers/index.html).
2. Considering the experimental setup of an on-chip PCR-based sequence detection and analysis system, the primary goal is to achieve hundreds of primer sequences that perform equally well at a common melting temperature and cycling conditions. Primer sequences for target amplification in the liquid phase must meet all requirements for multiplex primers as with conventional PCR. For instance, they have to be carefully checked for potential dimer formation with other primer sequences in the reaction mixture. A step-wise, empirical optimization might be required in some cases, helpful discussions of designing multiplex PCR protocols have been published by others ***(22–24)***.
3. The initial design of the arrayed oligonucleotides is easier because it allows a choice of between three to four possible primers for the sense and anti-sense strand, depending on the position of the target nucleotide relative to the 3´ end. Nested primer sequences for the solid-phase PCR should be selected for maximum allelic discrimination power, which sometimes has to be achieved empirically. In general, 3´ mismatches that are reported to be less refractory, such as G-T mismatches ***(25,26)***, should be avoided as well as high GC content at the 3´ termini. The length of the solid-phase primers should be adjusted to assure a common melting temperature. Note that the calculated, hypothetical melting temperature is higher than the empirically observed, due to the phenomenon of on-array melting temperature depression ***(27)***.
4. The nature and quality of glass slides and surface coating is a highly critical issue in on-chip PCR. The HCl step is important because it prepares the Si-OH layer of the glass surface required for chemical attachment of the silane. Optically flat and/or ultra clean substrates, as available from many microarray vendors, are not necessary for on-chip PCR. It is, however, very important that the slides are visually free of dust, carton residuals, oil, and fingerprints. Slides must be carefully

checked and, if required, cleaned using a precision cleaner such as the anionic detergent Alconox.

5. In our initial studies we screened a multitude of commercially available microarray glass slides and found that only slides containing a polymer-type surface coating in addition to the silane layer were suitable for on-chip PCR. The polymer layer increases the spatial distance between the glass-surface and the PCR primers, which decreases steric hindrance normally observed with DNA microarray surfaces ***(28)*** and enhances interaction between primers, template DNA, and polymerase at the primer binding region ***(13,29)***. Furthermore, we found that the attachment chemistry creating stable, covalent amide bonds between the polymer and the 5´ amino group of PCR primers is particularly compatible with the high temperatures during PCR. Thus, if combined with EGS crosslinker chemistry, other microarray glass slides containing a polymer-type surface coating are suitable for on-chip PCR, such as dendrimer coatings ***(30,31)*** or the commercially available CodeLink Activated Slides (Amersham Biosciences).
6. We observed that the concentration of the oligos to be spotted could range between 15 and 25 µ*M*. Although lower concentrations typically yield lower binding efficiencies, higher spotting concentrations also can result in lower efficiencies as a result of the electrostatic blockage of target hybridization at high surface probe density ***(27)***. Adjustment of spotting concentrations, however, might be required if other slide surfaces and/or other spotting technologies are used.
7. In addition to the Affymetrix spotter, which is a contact printer based on the so-called pin-and-ring technology, we also used a spotting robot that employs the widely used split-pin technology (TeleChem SMP 3 Stealth Pins). We advise one to carefully adjust spotting buffer, additives influencing surface tension such as SDS or DMSO, and spotting conditions like temperature and humidity according to the type of spotting technology used. Manufacturers of microarraying machines and substrates typically provide protocols for an optimal operating range regarding the aforementioned parameters.
8. The microarray layout should be designed carefully to allow simultaneous testing with several allele-specific primers. It is wise to array at least three replicates across several identical subarrays for each solid-phase oligonucleotide. Also, include guide dots that contain, for instance, a biotinylated oligonucleotide and can serve as controls for spotting, coupling, and staining (*see* **Fig. 1C**). Ideally, the subarrays are spotted with physically different pins of the spotting robot to control for potential differences and variations in deposited material or spot morphology. A "high-replicate" array pattern also provides auxiliary security in the event of possible technical troubles, such as scratches on the slide surface introduced during the handling of the slides. Moreover, we have demonstrated that replicates greatly facilitate downstream automated analysis steps in on-chip PCR like grid placement and spot finding ***(16)***.
9. Whenever possible, spotted slides should be stored at least 2 wk if the full binding capacity of the slide surface is needed. It is wise to produce the slides in batches of 40 to 80 pieces because the spotted slides can be stored at least 5 mo without loss of activity.

10. The technical challenge lies in performing a PCR in a small volume that is spread in a very thin layer over an area of 22 × 22 mm between the glass slide and the glass cover slip. For satisfactory efficiency, the enzymatic reaction performed on a flat glass surface requires a number of additives to the reaction mixture. First, the reaction is performed under higher salt (300 m*M* KCl, 50 m*M* Tris) and magnesium concentration (3 m*M*) compared to conventional PCR and contains bovine serum albumin as a blocking agent. Second, the reaction contains a self-seal reagent, which polymerizes upon contact with air at high temperature and thereby seals the reaction mixture at the edges to prevent evaporation during PCR. It is important to stick to this protocol and to check for the proper magnesium concentration that might require adjustment when using high primer, template or dNTP concentration. Virtually a single pipetting step is needed to launch the reaction from a complete master mix, which represents an important benefit with respect to potential sample contamination risk inherent to all PCR applications. A complete master mix can be prepared together with the polymerase and self-seal reagent in larger batches and stored in aliquots in lyophilized form after the addition of trehalose as stabilizer agent ***(32,33)***. After that, the reaction can be started by simply adding the DNA sample diluted to a 13 µL volume.
11. We have not quantified the actual amount of template DNA necessary for the on-chip PCR, but we normally obtain excellent results with template DNA extracted, for example, from overnight cultures of clinical swab samples using the following procedure. 750 µL of an overnight culture is centrifuged at 10,000*g* for 5 min and the supernatant is discarded. The pellet is then resuspended in 750 µL of PBS and centrifuged again. This second centrifugation and washing step yields better results, most probably as a result of the removal of possible PCR inhibitors ***(15)***. The pellet is finally resuspended in 200 µL of PBS and used for the DNA extraction procedure with commercial DNA extraction kits. It is important to note that template quality and quantity requirements in on-chip PCR are very similar to that in conventional tube PCR. It is advisable always to include positive control nucleic acid, such as DNA isolated from cultured reference strains, and negative control samples.
12. It is important that the cover slip that is used for sealing the reaction is very clean. Either specially treated cover slips for microarray applications should be used (Amersham Biosciences) or standard cover slips should be cleaned by ultrasonic treatment in detergent. Alternatively, the reaction could be performed under frame seals that are offered by some microarray vendors (MJ Research). Such self-adhesive frames might be beneficial under certain circumstances, although the reaction volume needed rises to a 25 µL minimum.
13. We previously found that denaturing washing steps after the amplification procedure is not necessary, suggesting that no significant nonspecific hybridization of unbound PCR products to un-reacted solid-phase primers occurs ***(13)***. Under certain circumstances, however, further stringent washing steps at high temperature could be useful to increase specificity.
14. Other dyes could be used, as well as the direct incorporation of commonly used Cy3 or Cy5-modified dCTP nucleotides. However, we have sometimes observed

higher background, most probably because of the denaturing effects on the glass surface of the fluorescence labels during thermal cycling. It should also be known that Cy5 dyes tend to degrade under ozone exposure ***(34)***.

15. Fluorescence intensities usually are calculated as spot medians after local background subtraction. Data analysis and interpretation can be substantially facilitated and automated by using scripting extensions. The Genepix software allows scripting extensions that can be used for automatically calculating discrimination scores from average values derived from the replicate spots. Automated routines for genotype assignment for bi-allelic variants have been described recently ***(17)***. The method is of course compatible with any other commercially available microarray slide scanner and accompanying data analysis software.

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4

An Array Biosensor for Detection of Bacterial and Toxic Contaminants of Foods

Miriam M. Ngundi and Chris R. Taitt

Summary

The Naval Research Laboratory has developed an array-based biosensor system capable of detecting multiple pathogenic and toxic species in complex matrices. Sandwich fluoroimmunoassays are performed on the surface of a patterned microscope slide that acts as an optical waveguide. Fluorescence from immunocomplexes formed on the slide surface is excited using the evanescent field, an electromagnetic component of light, and the pattern of fluorescence is imaged using a charge-coupled device camera. Using the evanescent wave for excitation allows real-time imaging. Alternatively, a confocal scanner can also be used to detect and quantify fluorescent spots. A method for immobilizing capture antibodies, performing assays, and detecting bound targets is presented.

Key Words: Immunoassay; immunosensor; antibody-based detection; food-borne contaminants; rapid detection; *Salmonella*; *Campylobacter*; staphylococcal enterotoxin B; SEB.

1. Introduction

A biosensor is a device that uses biological recognition molecules to detect and identify a target with high selectivity and sensitivity. The most commonly used biological molecules in sensor technology are antibodies because of their selective nature toward their target (antigen). The immunological basis of immunosensors makes them highly specific, often highly sensitive, and not dependent on sample cleanup or preparation. In recent years, there has been advancement in the development and application of biosensor technology using two-dimensional arrays ***(1–4)***. The ability of an array-based biosensor to simultaneously analyze multiple samples for multiple analytes makes it superior over

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other types of biosensors, enabling multiple controls and internal standards to be analyzed in parallel with unknown samples ***(2,3,5,6)***.

The array biosensor developed at the Naval Research Laboratory (NRL) is rapid and capable of simultaneous analysis of samples for multiple analytes ***(2,5–8)***. The current version of the array biosensor is portable, weighing less than 10 lb, is fully automated (*see* **Fig. 1A [*9–12*]**), and has the potential to be used as a screening and monitoring device for clinical, food, and environmental samples ***(11,13–17)***. The system consists of an array of immobilized and soluble biological-recognition elements, fluidics components, an optical transducer, and data-analyzing software. The biological-recognition elements consist of "capture" species that are covalently immobilized to the sensor substrate and fluorescently labeled "tracer" antibodies in solution. The immobilized "capture" biomolecules form parallel patterns of either array stripes or microarrays ***(7,18)*** on a glass slide, which serves as a waveguide. Multiple samples are simultaneously applied onto the slide orthogonal to the patterned "capture" species and subsequently detected directly or indirectly using a fluorescent-labeled "tracer" antibody. The fluorescent antibody complex bound to the surface of the slide is excited at 635 nm, and the fluorescent emission from the slide is imaged onto a CCD imaging array (*see* **Fig. 1B**).

Four different assay formats: direct, displacement, competitive, and sandwich assays can be performed with the array biosensor ***(19)***. Direct and displacement assay formats have limitations associated with their application in real sample analysis and are therefore used less commonly in real-world applications. A competitive assay format is used for the detection of small molecular weight analytes, such as mycotoxins ***(13)***, in which the antigen or analyte does not possess two distinct epitopes to which the capture and tracer antibodies can bind simultaneously. In competitive assays, an analog of the analyte is covalently attached onto the slide to serve as the capture species. Sample solutions (containing unlabeled analyte) are spiked with a constant concentration of fluorescent-labeled antibody and are passed over the patterned slide. The (unlabeled) analyte in solution competes with the immobilized analyte for binding on the antibody. Binding of antibody to the immobilized analyte produces a fluorescent antibody–analyte complex, whose signal is inversely proportional to the concentration of the analyte in the sample ***(1,13)***.

In sandwich assays, the immobilized "capture" molecules are incubated with the samples, followed by a solution containing fluorescent "tracer" antibody. Typically, antibodies are used for both "capture" and "tracer" elements, although other molecules have been used ***(20)***. The sandwich assay forms a capture–analyte–antibody complex on the surface of the slide. The fluorescent signal of the complex is directly proportional to the amount of analyte in the sample. This chapter describes in detail the protocols followed in performing a sandwich assay using the NRL array biosensor.

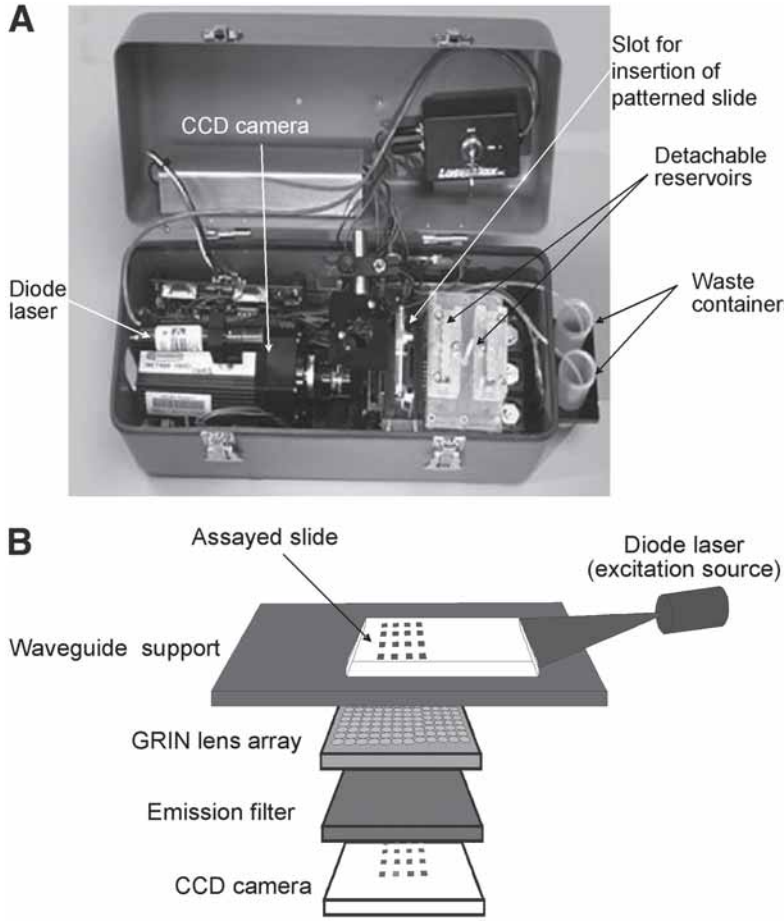

Fig. 1. **(A)** The current version of the portable array biosensor. **(B)** A schematic diagram of the optical system used in the array biosensor

The optical system of the array biosensor has been described in detail by Feldstein et al. ***(21)*** and by Golden and Ligler ***(22)***. It comprises a 635-nm, 12-mW diode laser excitation source, a waveguide support, GRIN lens, several emission filters, and a Peltier-cooled charge-coupled device (CCD) imaging array (*see* **Fig. 1B**). An excitation beam is launched into the edge of the waveguide at an appropriate angle (approx 36°), resulting in the evanescent excitation of the surface-bound fluorescent antibody–antigen complexes. The fluorescent emission produced by the complexes is imaged onto the CCD. The digital images of the waveguide are acquired in the Flexible Image Transport System (i.e., FITS) for-

mat, and data in the form of fluorescent intensities are extracted from the images using a custom data analysis software program ***(10,20,23)***. The software program generates a mask that consists of square or rectangular data spots (corresponding to the area where the capture species were exposed to the detection antibody) and background rectangles on either side of the data spot. The net fluorescent intensity for each data spot is obtained by subtracting the average background value from the data spot value. These values are imported into Microsoft Excel files and analyzed.

2. Materials

1. Cleaning solution: 10% (w/v) KOH in MeOH. This solution should be handled with care because it is very corrosive and dissolution process is exothermic. Isopropanol may also be used in place of MeOH.
2. Silane–toluene mix: 1 mL of 3-mercaptopropyl tri(m)ethoxy silane (Fluka) in 49 mL of anhydrous toluene. Toluene is a hepatotoxin and a reproductive hazard. All solutions containing toluene should be used inside a chemical hood and a respirator with appropriate filtration system used. The glovebag used for preparation of this solution and silanization of the slides must be mounted within a chemical fume hood with appropriate spacing to allow airflow. 3-Mercaptopropyl tri(m)ethoxysilane is sensitive to moisture and light and must be stored at 4°C in a desiccator chamber (*see* **Note 1**).
3. GMBS/EtOH: *N*-succinimidyl-4-maleimidobutyrate (GMBS; Pierce, Rockford, IL) in 43 mL of absolute ethanol. Dissolve GMBS in 0.25 mL of anhydrous dimethylsulfoxide (DMSO) before adding it to ethanol. Use solution immediately after preparation. GMBS is sensitive to moisture and must be stored in a desiccator at 4°C. Store opened stock bottle of EtOH in glovebag.
4. NeutrAvidin solution: 1 mg of NeutrAvidin (Pierce) in 33 mL of phosphate-buffered saline (PBS), pH 7.4. Aliquots of 1 mg/mL NeutrAvidin in PBS can be made up in quantity and stored at 4°C or frozen until use.
5. PBS, pH 7.4.
6. PBSTB: PBS containing 0.05% (w/v) Tween-20 and 1 mg/mL bovine serum albumin (BSA).
7. Blocking solution: 10 mg/mL BSA in 10 m*M* sodium phosphate buffer, pH 7.4.
8. Antibodies: rabbit anti-*Salmonella* sp. and rabbit anti-*Campylobacter* (Biodesign International), rabbit, and sheep anti-staphylococcal enterotoxin B (SEB; Toxin Technology, Inc.), mouse monoclonal anti-SEB, clone 2B (BioVeris); biotin-conjugated rabbit anti-chicken IgY, and Cy5-chicken IgY (Jackson ImmunoResearch).
9. Antigens: heat-killed *Salmonella typhimurium* and *Campylobacter jejuni* (KPL); SEB (Toxin Technology, Inc.). These are reconstituted according to the supplier's instructions and store at 4°C.
10. Biotin: EZ-link NHS-LC-biotin (Pierce). This biotin conjugate is sensitive to moisture and must be stored desiccated at 4°C.

11. Cy5-dye: Cy5 Bisfunctional Reactive Dye (Amersham Life Science). Cy5 dye is light-sensitive and should be stored in the dark at 4°C. Once each vial is opened and dissolved in anhydrous DMSO, it must be used within several hours.
12. "Capture" antibodies: Biotin conjugates of rabbit anti-*Salmonella*, rabbit anti-*Campylobacter*, rabbit or monoclonal anti-SEB. Store at 4°C. Concentrations of 10 or 20 µg/mL in PBS are used for pattering. *See* **Subheading 3.2.1.** for biotinylation protocol.
13. "Tracer" antibodies: 10 or 20 µg/mL in PBSTB of Cy5 labeled with each of rabbit anti-*Salmonella*, rabbit anti-*Campylobacter*, and sheep anti-SEB. Cy5 conjugates are sensitive to light; therefore, they must be stored in dark at 4°C. *See* **Subheading 3.3.2.** for Cy5 labeling of antibodies.
14. Borosilicate microscope slides (3-in. × 1-in.; Daigger Vernon Hills, IL).
15. Poly(dimethylsiloxane) (PDMS) flow channels are prepared from liquid silicone elastomers (Nusil Technology, Carpinteria, CA).

3. Methods

3.1. Attachment of NeutrAvidin to the Slide Surface

A nonglycosylated derivative of avidin, NeutrAvidin, is covalently attached to the surface of the microscope slide to create a generic surface suitable for creation of patterns of immobilized biotinylated capture antibodies. The cleaned surface is first treated with a thiol silane, followed by a heterobifunctional crosslinker possessing thiol-specific and amine-specific moieties. After the thiol-specific (maleimide) moiety is reacted with the –SH-derivatized slide, the amine-specific (*N*-hydroxysuccinimidyl ester) terminus of the crosslinker is reacted with amines on lysine residues on the NeutrAvidin, thereby covalently attaching the protein to the surface.

3.1.1. Cleaning

The purpose of the cleaning step is to remove organic compounds and other contaminants from the surface of the glass slide and expose hydroxyl groups required in silanization process ***(24)***.

1. With a carbide-tipped pencil, etch an appropriate identifier in the upper left corner of each slide to orient and identify the slide.
2. Place slides back-to-back within a coplin jar (16 slides/jar).
3. For each batch of 16 slides, prepare 100 mL of KOH/MeOH cleaning solution. Weigh 10 g of KOH in a flask and add 100 mL of MeOH. Use a stir bar to stir until all the KOH has dissolved
4. Pour over the slides and incubate for 30 min at room temperature.
5. Carefully remove each slide and rinse exhaustively under distilled water until no schlieren lines are observed. Immerse cleaned slides in distilled water in a clean Coplin jar. Then, dry each slide under a stream of nitrogen.
6. Once dried, place slides back-to-back in clean, dry Coplin jar.

3.1.2. Silanization

During this process, the hydroxyl groups on the surface of the slide react with the ethoxy groups of 3-mercaptopropyl tri(m)ethoxy silane, resulting in a thiol-derivatized slide. This step, and all subsequent silanization steps, must be performed inside a glove-bag or other nitrogen-filled environment.

1. For each batch of 16 slides, prepare 50 mL of silane–toluene mix immediately before use (*see* **Note 1**). Pour the silane–toluene mix over the slides and incubate, under nitrogen atmosphere, for 1 h.
2. Remove the Coplin jar from the glovebag and place elsewhere in chemical fume hood.
3. With forceps, remove each slide from silane–toluene solution and rinse three times with toluene by swishing the slides three to five times sequentially in three 150-mL beakers filled with toluene.
4. After the third wash, apply a nitrogen stream to the surfaces of the slide to remove residual toluene. Ensure that toluene vapors and droplets are evaporated within the hood.
5. Place slides back-to-back in a clean, dry Coplin jar. Proceed immediately with crosslinking.

3.1.3. Crosslinking and Attachment of NeutrAvidin

During this step, the thiol groups on the surface of the slide react with the maleimide moiety of GMBS and the *N*-hydroxysuccinimidyl ester terminus of the crosslinker is later reacted with amines on lysine residues on the NeutrAvidin. This step results in NeutrAvidin-coated slides. Crosslinking and all subsequent steps may be performed outside of the chemical hood, on the bench top.

1. For each batch of 16 slides, prepare 43 mL of GMBS/EtOH mix immediately before use.
2. Pour GMBS/EtOH mix over slides and cover the jar to minimize the evaporation of EtOH. Incubate for 30 min at room temperature.
3. During this incubation, prepare NeutrAvidin solution and pour into fresh, clean Coplin jar.
4. With forceps, remove each slide from GMBS/EtOH mix and rinse three times with water by swishing the slide three to five times sequentially in three 150-mL beakers filled with water.
5. After the third rinse, place slide immediately into Coplin jar containing NeutrAvidin solution, ensuring that slides are oriented back-to-back.
6. Incubate slides in NeutrAvidin solution overnight at 4°C.
7. Rinse each slide three times in PBS and store in PBS at 4°C (*see* **Note 2**).

3.2. Patterning of Biotinylated "Capture" Antibodies

Biotinylated "capture" antibodies are loaded into channels of a PDMS patterning template and incubated overnight. After removal of the patterning

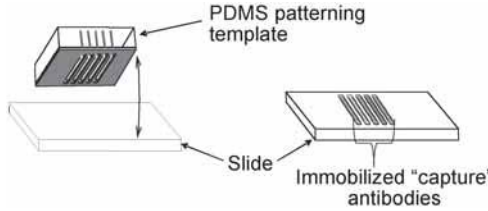

Fig. 2. The poly(dimethylsiloxane) (PDMS) patterning template is pressed against a NeutrAvidin-coated slide. Solutions of "capture" antibodies are loaded onto the channels and incubated overnight. After removal of the solution and template, stripes of "capture" antibody are patterned on the slide.

solution(s), the channels are rinsed with buffer, and the patterning template removed, leaving a series of stripes of immobilized antibodies (*see* **Fig. 2**).

3.2.1. Biotinylation of "Capture" Antibodies

This protocol may be scaled up or down to accommodate smaller or large volume of antibody stock solution.

1. Calculate the amount of biotin-LC-NHS ester needed to for 5:1 (biotin:antibody) ratio.
2. Dilute antibody solution such that the final concentration is 1 to 2 mg/mL (*see* **Note 3**).
3. Add 1/9 volume of 0.5 *M* bicarbonate buffer, pH 8.5, to the antibody solution such that the final bicarbonate buffer is 50 m*M*.
4. Dissolve biotin-LC-NHS ester in DMSO to a final concentration of 1 mg/mL.
5. Add the biotin–DMSO mix to the diluted antibody in bicarbonate buffer, such that the final biotin to antibody ratio is 5:1.
6. Incubate the antibody/biotin mix at room temperature for 30 min while rocking.
7. Pipet mix onto a Bio-Gel P-10 column, which has been pre-equilibrated in PBS (*see* **Note 4**). Allow the sample to soak into gel. Rinse top of gel and sides of column with PBS.
8. Add a layer of 1 to 5 mL of PBS onto the top of gel and monitor the absorbance of the eluent at 280 nm. Collect all eluent by fractions and save the first peak.
9. Add more PBS buffer as necessary.
10. Dilute an aliquot of the first peak fraction with PBS (typically 10× dilution) and measure the absorbance at 280 nm.
11. Determine the concentration of the biotinlylated conjugate (*see* **Note 5**) and store at 4°C.

3.2.2. Preparation of PDMS Patterning and Assay Templates and Mounting Manifolds

Template molds are milled as a positive relief in Plexiglas or other plastic material. Typically, the positive relief channels are 21 mm (l) × 1 mm (w) × 2.5

mm (h) for patterning templates and 40 mm (l) × 1 mm (w) × 2.5 mm (h) for assay templates ***(21)***.

1. Weigh out 200 g of Nu-Sil MED-4011, Part A, in a large disposable plastic beaker (approx 1 L volume; *see* **Note 6**).
2. Weigh out 20 g of Nu-Sil MED-4011, Part B, component and add to Part A component.
3. Mix well with a disposable applicator stick until translucent lines are observed.
4. Transfer beaker to vacuum chamber and apply vacuum until all bubbling has ceased (*see* **Note 7**).
5. Transfer PDMS mix to template molds, avoiding bubbles whenever possible.
6. Place template molds into vacuum chamber and apply vacuum until bubbling has ceased.
7. Remove template molds from vacuum chamber and heat for 30 min at 65°C.
8. Incubate at room temperature for 3 d before removing from mold.

Mounting manifolds are manufactured for reproducible placement of the patterning and assay templates on the slides. These manifolds are milled from Plexiglas or another plastic. A lower piece is milled with a single groove to hold the slide in place. Upper pieces are milled to possess two openings to allow the insertion of syringes into the PDMS templates ***(21)***.

3.2.3. Patterning of "Capture" Antibodies

The goal of this step is to introduce the biotinylated antibodies onto the surface of the NeutrAvidin-coated slides. The antibodies become immobilized onto the surface of the slide.

1. Remove NeutrAvidin-coated slide from PBS and dry briefly under a nitrogen stream.
2. Place slide face-up into slot of lower patterning manifold.
3. Place PDMS patterning template on surface of NeutrAvidin-coated slide with channels facing the surface of the slide.
4. Place upper (patterning) manifold on top of PDMS piece and tighten into place.
5. For each antibody solution, fill a 1-mL syringe with the appropriate solution and insert needle into one end of each channel. Insert an open syringe barrel into the other end of the channel as an outlet (*see* **Note 8**).
6. Repeat injection of appropriate antibody solutions into each channel until all channels are full.
7. Remove all syringes. Incubate overnight at 4°C.
8. The following day, connect one end of each channel to (multichannel) peristaltic pump using a syringe (outlet). A single channel peristaltic pump may be used in the absence of a multihead pump.
9. Insert a syringe into the opposite end of each channel and attach to an empty syringe barrel reservoir (inlet). **Figure 4** illustrates the assembled slide during assay.
10. Start the pump at a 1 mL/min flow rate, applying suction from the outlet.
11. Allow each channel to fill with air and stop the pump.

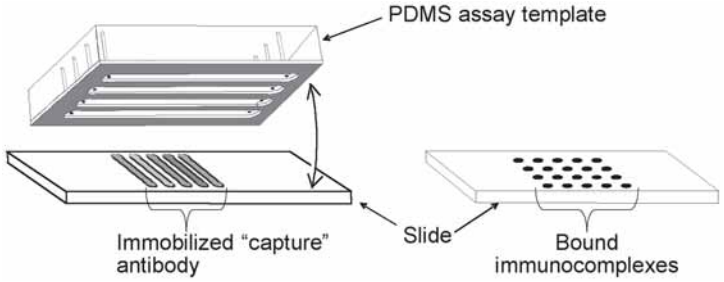

Fig. 3. The poly(dimethylsiloxane) (PDMS) assay template is pressed against a patterned slide. After assay, "spots" of "capture" antibody–antigen–"tracer" antibody complexes are bound where assay solutions have interacted with "capture" antibody.

12. Pipet 1.0 mL of PBSTB into each reservoir and restart the pump at a 1-mL/min flow rate. Allow each channel to rinse with PBSTB and finally evacuate.
13. Remove all fluidics connections from the patterning template and disassemble patterning template sandwich.
14. Place patterned slide into blocking solution and incubate for 30 min.
15. Dry the patterned, blocked slides under nitrogen stream. Slides can be stored at 4°C for as long as 5 mo ***(11)***.

3.3. Assay

Extracts of food samples spiked with the analyte of interest are loaded into channels of PDMS assay template and are passed over the patterned slides for approx 10 to 15 min. The protocol described uses spiked samples but is equally applicable to naturally contaminated samples. The unbound analytes are rinsed off and fluorescently labeled antibody ("tracer" antibody) is passed over the slide for 4 min. Bound immunocomplexes are formed at areas in which the assay solutions interact with patterned "capture" antibody (*see* **Fig. 3**). The fluorescently bound antibody complex is detected using a CCD camera as described in **Subheading 3.4**. Assay protocols are first developed and optimized in PBSTB before being applied to the food samples. During optimization, the appropriate concentrations of the "capture" and the "tracer" antibodies to be used for the assay are determined. The array biosensor can be employed for detection of analytes based on a competitive assays format (*see* **Note 9**).

3.3.1. Cy5 Labeling of "Tracer" Antibody

The following protocol is appropriate for labeling 1 mg of antibody. It may be scaled up or down to accommodate smaller or larger amounts of antibody. Buffers containing primary amines such as Tris and glycine will inhibit the conjuga-

tion reaction. Therefore, antibody stocks in these buffers must be subjected to a buffer exchange process, preferably with PBS.

1. Dilute 1 mg of antibody with PBS to a final protein concentration of 1 to 2 mg/mL (use a 1.5- or 1-mL centrifuge tube).
2. Add 1/9 volume of 0.5 *M* borate buffer, pH 8.5, such that the final borate buffer concentration is 50 m*M*.
3. Dissolve 1 vial of Cy5-NHS-ester in 50 µL of DMSO immediately before adding to the antibody (*see* **Note 10**).
4. Add 15 µL of Cy5/DMSO mix to the antibody.
5. Wrap the vial with aluminum foil and incubate at room temperature for 30 min while rocking (*see* **Note 11**).
6. Pipet mix onto a 25 mL of Bio-Gel P-10 column that has been pre-equilibrated in PBS (*see* **Note 4**). Allow the sample to soak into gel and rinse top of gel and sides of column with PBS.
7. Add PBS onto of the top of gel, collect and save the first blue fraction. Store in the dark.
8. Take appropriate volume of the eluted first blue fraction and dilute with PBS (typically 10× dilution).
9. Take the absorbances at 280 nm and 650 nm. Determine the concentration of the antibody and the dye to protein ratio (*see* **Note 12**). Store Cy5-labeled antibody in the dark at 4°C.

3.3.2. Preparation of Food Samples

Each food is prepared differently depending on its texture, with the goal of making it homogenous enough to flow through the syringes and tubing at a 0.1 mL/min flow rate. Protocols detailed are based on those used by the US Food and Drug Administration, the Center for Food Safety and Applied Nutrition, and the US Department of Agriculture ***(25–27)***.

3.3.2.1. Ham, Ground Beef, Pork Sausage, Cantaloupe

1. Weigh out several aliquots of each food (1–10 g).
2. Add equal volume of PBSTB containing various concentrations of the analyte.
3. Place the food–buffer mixture (with or without analyte) into a Waring blender and homogenize at high speed for 2 min.
4. Transfer the homogenate samples into centrifuge tubes (15-mL volume) and centrifuge at 3000*g* for 10 min.
5. Analyze the supernatant.

3.3.2.2. Chicken Carcass Wash

1. Place a fresh chicken carcass and 100 mL of PBS containing 1 mg/mL of BSA in a resealable bag (e.g., Ziplock®).
2. Seal the bag and incubate for 2 h at room temperature on a rocking platform.
3. Remove the liquid (carcass wash) and spike aliquots with analyte (*see* **Note 13**).
4. Analyze without further treatment.

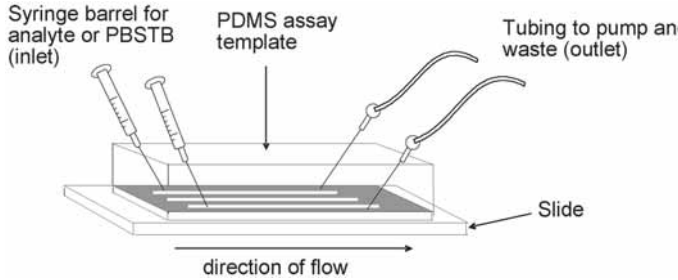

Fig. 4. Schematic diagram of the assembled patterned slide and fluidics during assay. The upper and lower manifolds are not shown.

3.3.2.3. Egg

1. Blend whole eggs until in liquid form and aliquot into the appropriate volumes.
2. Added equal volume of PBSTB containing various concentrations of analyte.
3. Transfer the egg–PBSTB mix into a Waring blender and mix on high speed for 2 min.
4. Analyze the diluted homogenate without further treatment.

3.3.3. Assay Protocols

Sample analysis is performed on the "capture" antibody-patterned slides using the PDMS assay template and mounting manifolds described in **Subheading 3.2.2.** However, for assays, the upper piece of the assay-mounting manifold has the two openings for needle insertion perpendicular to those for patterning.

1. Place patterned slide face-up into slot of lower assay manifold (same manifold used for patterning).
2. Place PDMS assay template on the surface of patterned slides, with channels facing the slide and orthogonal to the stripes of the immobilized "capture" antibody.
3. Place upper assay manifold on top of the PDMS and tighten into place with screws.
4. Insert needle with an open barrel into one end of each PDMS channel (inlet; *see* **Fig. 4**).
5. Insert needle attached to tubing of a (multichannel) peristaltic pump into the other end of each PDMS channels (outlet; *see* **Note 14**).
6. Pipet 1.0 mL of PBSTB into each syringe barrel reservoir and start the pump at a 1 mL/min flow rate. After the PBSTB has flowed through each channel and reservoir is empty, allow each channel to fill with air and stop the pump.
7. Pipet 0.8 mL of the prepared food sample containing the various concentrations of analytes into the syringe reservoirs. Include a buffer blank (0.8 mL of PBSTB). Restart the pump at a 0.1 mL/min flow rate (*see* **Note 15**).
8. Allow the assay to run for 15 min then empty the channels. Allow them to fill with air and stop the pump.

9. Pipet 1 mL of PBSTB into the syringe reservoir and start the pump at a 1 mL/min flow rate. After approx 1 min, allow each channel to fill with air and stop the pump (*see* **Note 16**).
10. Pipet 0.4 mL of the "tracer" antibody into each reservoir, start the pump at a 0.1 mL/min flow rate. After the "tracer" has flowed through each channel and reservoir is empty, allow each channel to fill with air and stop the pump.
11. Pipet 1 mL of PBSTB into the syringe reservoir and start the pump at a 1 mL/min flow rate. After approx 1 min, allow each channel to fill with air and stop the pump.
12. Remove all fluidics connections from the assay template and disassemble assay template.
13. Rinse the entire slide with water and dry under a stream of nitrogen.
14. Image slide immediately or store in the dark for up to 24 h for later imaging.

3.4. Imaging and Data Analysis

Detailed description of the imaging system is given by Feldstein et al. **(*21*)** and by Golden and Ligler ***(22)***. Alternatively, a confocal scanner may be used.

1. Turn on the laser light and allow it to stabilize for 5 min.
2. Place the slide with completed assays face-up on the waveguide holder.
3. Choose the appropriate exposure time and collect the images.
4. Analyze data using appropriate image analyzing program ***(21,23)***.

Figure 5 demonstrates the results obtained when several analytes (*Salmonella*, *Campylobacter*, and SEB) were analyzed simultaneously using the same substrate. In this case, the slide was patterned with the three "capture" antibodies (three lanes each), PBS as negative control, and rabbit antichicken IgY as positive control. Serial dilutions of the analytes in PBSTB were assayed and a CCD image of the fluorescently-bound complex taken with a 1.5-sec exposure time. The fluorescent intensities showed dose dependence for each analyte. Even though this particular image did not show dose dependence for SEB, images taken using a shorter exposure time showed dose-dependent fluorescent signal intensities. The variation in the fluorescent intensities observed for SEB "captured" using monoclonal versus polyclonal antibodies illustrate the differences in specificities for these antibodies towards SEB.

4. Notes

1. Aliquotting of this reagent (under nitrogen) into small (<3 mL) amber vials is recommended. Allow the desiccator chamber to come to room temperature before opening. Likewise, allow each aliquot to come to room temperature. After opening a stock bottle of toluene, store it in a glovebag. Prepare the silane–toluene mix immediately before use. Silane–toluene mix forms a polymerized chemical layer on glassware. All glassware used during silanization must be rinsed with acetone (store the waste in a suitable chemical waste container in the hood), then in water.

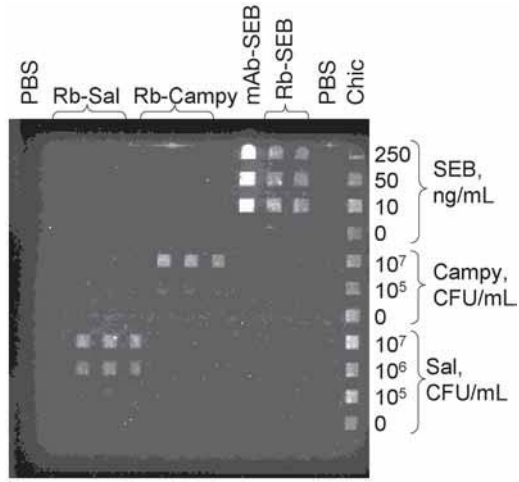

Fig. 5. Charge-coupled device image of a sandwich assay for *Salmonella*, *Campylobacter*, and staphylococcal enterotoxin B (SEB) at a 1.5-s exposure time. The slide was patterned with 20 µg/mL of biotinylated rabbit antibodies against *Salmonella* (Rb-Sal), *Campylobacter* (Rb-Campy), and SEB (Rb-SEB); 10 µg/mL biotinylated monoclonal antibodies against SEB (mAb-SEB); and Biotin-SP-conjugated AffiniPure rabbit anti-chicken IgY (Chic) in phosphate-buffered saline. Samples were spiked with SEB, *Campylobacter* (Campy), or *Salmonella* (Sal), indicated to the right of the image. "Tracer" antibodies consisted of 20 µg/mL Cy5-labeled rabbit anti-*Salmonella*, 20 µg/mL Cy5-labeled rabbit anti-*Campylobacter*, and 10 µg/mL Cy5-labeled sheep anti-SEB, each containing 100 ng/mL Cy5-conjugated ChromPure chicken IgY.

Glassware is then incubated overnight in a base bath and finally rinsed exhaustively with water.

2. NeutrAvidin-coated slides stored at 4°C are stable for as long as 5 mo ***(11)***.
3. As the reaction chemistry links biotin (or Cy5) to the antibody via amine groups, the antibody must be in a buffer that does not contain amine moieties. Amine-based buffers (e.g., Tris, glycine) must be removed from the antibody prep prior to the labeling reaction. Any method suitable for desalting may be used for buffer exchange.
4. The Bio-Gel P-10 column is prepared by first suspending Bio-Gel P-10 gel (medium, 90–180 µm) into PBS to make a slurry. To completely hydrate the Bio-Gel, allow the slurry to sit overnight at room temperature or alternatively, autoclave or boil for 20 min. The hydrated slurry is stable at room temperature for months. Immediately before loading the column, the slurry is degassed by application of a vacuum. The slurry is then loaded into a 25-mL column prefilled with approx 3 mL of PBS. Once the column has been filled, it is then flushed with at least three volumes of

PBS. After the elution of the conjugate products, the column must be flushed exhaustively with PBS and stored wet (PBS) at room temperature for future use.

5. The absorbance at 280 nm should be below a value of 1.0 absorbance units for accurate determination of concentration. The concentration of biotinylated antibody is determined by the Beer Lambert Law ($A = \varepsilon cl$), with $\varepsilon_{280\ nm,1mg/ml,\ 1cm} = 1.4$.
6. Each component of the silicon elastomer is very sticky. It is recommended that a paper towel be used to cover the balance as well as the bench top on which the beaker and stock containers are placed.
7. Once the vacuum is applied, the mix must be observed until the rising contents start to drop down (if overflow occurs, the vacuum must be stopped). It is recommended that a paper towel be placed inside the vacuum chamber prior to placing the beaker.
8. It is recommended that positive controls, such as anti-chicken IgY, be patterned at the outmost channels and with the buffer blank (negative control) channels immediately adjacent. This format prevents any interference of the analyte signals in the event of leakage due to faulty contact with the PDMS patterning template. The positive control channels should be the last to be patterned.
9. The patterning protocol is similar to that described in **Subheading 3.2.3.,** except that the biotinylated analog of the analyte is used as the capture molecule. The samples are prepared accordingly and a constant concentration of the Cy5-labeled antibodies against the analyte (and Cy5 labeled positive control) is added to each sample ***(13)***. These then are assayed for 15 min. Initially, a checkerboard-type of assay, whereby different concentrations of capture molecules are exposed to various concentrations of Cy5-labeled "tracer" antibody in PBSTB, is performed to determine the reasonable working concentrations for both capture molecules and "tracer" antibody.
10. The Cy5-dye in DMSO is stable for several hours, provided that anhydrous DMSO is used. Otherwise, it is stable for approx 30 min. As only 15 µL of the dissolved Cy5 is used to label 1 mg of antibody, each vial may be used to label multiple batches of antibody, as long as all labeling reactions are performed within a period of several hours. Cy5-dye is light-sensitive and all Cy5 solutions must be protected from light.
11. Incubation time may be extended to increase the dye to protein ratio. However, for optimal labeling efficiency the molar ratio should be maintained at 2:1 to 4:1 because higher ratios have been shown to exhibit quenching characteristics ***(28)***.
12. The absorbance at 280 and 650 nm (A_{280} and A_{650}, respectively) must be less than 1 absorbance unit for accurate determination of concentrations. The concentration of the Cy5-labeled antibody (moles/liter) is given by: $[A_{280} - (0.05 \times A_{650})] / 170{,}000$. The dye to protein ratio is calculated by: $(0.68 \times A_{650}) / [A_{280} - (0.05 \times A_{650})]$.
13. The carcass wash can be stored frozen at –20°C for later analysis. Frozen carcass wash must be thawed before spiking with analyte.
14. As a result of bubbles that occasionally form at the ends of the patterning channels, it is recommended that one not use the outermost assay channels or not to run analyte samples on them. These outermost channels often produce incomplete "spots" (*see* **Fig. 5** uppermost spots).

15. Sensitivity may be improved by extending the duration in which the sample is exposed to the slide, which could be performed without increasing the volume by recirculating the sample over the slide.
16. The "tracer" species of the positive control is added to all "tracer" antibody solutions. If the analysis is geared toward a simultaneous analysis of *multiple* samples for *multiple* analytes, then the "tracer" antibody is a cocktail of all tracer antibodies plus the positive control.

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5

Detection of *Neisseria meningitidis, Streptococcus pneumoniae,* and *Haemophilus influenzae* in Blood and Cerebrospinal Fluid Using Fluorescence-Based PCR

Stuart C. Clarke

Summary

The polymerase chain reaction (PCR) is a fundamental part of modern molecular biology. Fluorescence-based PCR methods also are now available, which enable rapid, specific, and sensitive assays for the amplification and analysis of deoxyribonucleic acid (DNA). These methods are performed in closed-tube format, thereby reducing the risk of contamination between stages. In addition, post-PCR processing, such as clean-up steps and gel electrophoresis, are eliminated as the results are read via an integrated fluorimeter. An example of this methodology is fluorescence-based PCR using dual-labeled probes, termed dual-labeled end-point fluorescence PCR. This method uses oligonucleotide probes that are dual-labeled with a reporter dye and quencher dye. The method has the advantage that DNA extraction, liquid handling, PCR, and analysis also can be fully automated. In this chapter, the simultaneous detection of *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* from clinical samples is described.

Key Words: Polymerase chain reaction; fluorescence-based PCR; PCR; DNA amplification; *Neisseria meningitidis*; *Streptococcus pneumoniae*; *Haemophilus influenzae*; meningitis; septicemia; laboratory diagnosis; molecular biology.

1. Introduction

Molecular biology as a science was revolutionized in the late 1980s as a result of the development of the polymerase chain reaction (PCR) ***(1)***. For many years, PCR has been used extensively to enable the selective or nonselective amplification of deoxyribonucleic acid (DNA) so that further analyzes can be performed ***(2)***. Without it, DNA sequencing or DNA cloning would not be

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possible, and much of what we now know about eukaryotic and prokaryotic biology may not have been achievable. Since the initial description of PCR, there have been a plethora of applications and variant methods to further capitalize on the theory of PCR. Additional applications include reverse-transcriptase PCR (i.e., RT-PCR), enzyme-linked immunosorbent PCR (i.e., ELISA–PCR), and fluorescent PCR.

Several methods are available for the detection of specific nucleotide sequences ***(3,4)*** but some have disadvantages, such as low sensitivity, a lack of specificity, high cost, or laborious methodology ***(5)***. Fortunately, some of the shortcomings of traditional PCR have been recognized, and revisions to the method have been made. Some of the disadvantages may be avoided with the use of good laboratory practice, the use of careful assay design, and the inclusion of appropriate controls ***(6)***. Nonetheless, the principles of PCR have remained largely the same such that, for traditional PCR, a commercial thermocycler is used to amplify specific nucleotide sequences and the products are visualized by agarose gel electrophoresis. However, changes have been made to make the process quantitative, accurate, and less labor-intensive. Moreover, real-time PCR is now available, which means that the assay can be followed accurately during amplification rather than relying on end point analysis. Real-time PCR is performed in closed tube format, thereby reducing the risk of contamination between stages. Post-PCR processing, such as clean-up steps and gel electrophoresis, are eliminated because the results are read via an integrated fluorimeter ***(7)***.

Real-time PCR methods use chemistries, such as hydrolysis probes and hairpin probes, which are commercially available as TaqMan® and Molecular Beacons, respectively ***(4,8)***. They rely on the use of a homogenous fluorescent detection system based on the principle of fluorescent energy transfer (i.e., FRET), which was first described in 1978, to measure the extent of amplification ***(9,10)***. A probe is included in the assay reaction that posseses a 5´ fluorophore and a 3´ quencher molecule. Although the fluorophore and quencher remain in close contact, the quencher moiety transfers the fluorescent energy to heat, but when the fluorophore and quencher are not in close contact, the energy is emitted in the form of light. The amount of fluorescence emitted during real-time PCR is directly related to the quantity of DNA amplified ***(11)*** and is based on the threshold cycle (Ct), which is the cycle at which fluorescence is determined to be statistically significant greater than background fluorescence. These calculations are important because specific cut-off points can be used in clinical assays. Real-time PCR is thought to be more accurate than endpoint PCR because measurements are taken during the exponential phase of PCR and calculations derived from these. They will not be affected by

limiting reagents, small differences in reaction components, or cycling parameters ***(3)***.

In practice, such as in a clinical diagnostic laboratory, real-time PCR can be used in place of traditional methods for the detection and identification of bacteria and viruses ***(12–16)***. Nonculture confirmation of disease and characterization of the infecting organism also can be performed by real-time PCR ***(13,17)***. For bacterial infections in particular, the infecting bacterium can be identified to species level and further typed using fuorescence-based PCR. Such methods have been developed for meningococcal disease and currently are being developed for other important pathogens ***(15,17,18)***. However, clinical applications of commercially available fluorescence-based PCR methods currently are limited and remain relatively expensive for routine use. Therefore, assays designed and validated in research laboratories are required for particular purposes. An example of such method is fluorescence-based PCR using dual-labeled probes, which was termed dual-labeled endpoint fluorescence PCR (DEF-PCR ***[13,15, 17]***). Oligonucleotide primers are dual-labeled with a reporter dye, such as 6-carboxyfluorescein (FAM), covalently linked to the 5′ end and the quencher dye 6-carboxy-tetramethylrhodamine (TAMRA) to the 3′ end. As for real-time PCR, a probe hybridizes to a specific DNA sequence upon PCR product formation but is subsequently digested by 5′ exonuclease activity of Taq DNA polymerase during primer extension, thus releasing the reporter dye and increasing fluorescence emissions.

Automation also has recently become more affordable and is therefore accessible to more laboratories ***(2)***. It is now used heavily in the pharmaceutical industry and more recently in academic research and clinical diagnostics. Fluorescence-based PCR methods can therefore now be fully automated on a liquid-handling robot, including the steps for DNA extraction, liquid handling, and thermocycling, and the formation of PCR products analyzed via the alteration and subsequent increase in fluorescence emissions using an integrated 96-well format fluorimeter ***(15,19,20)***. The advantages of this methodology over traditional PCR are the capacity for high throughput and full automation.

Fluorescence-based PCR, whether real-time or endpoint, addresses many of the inadequacies of traditional PCR. The cost of setting up such a system can be expensive, although the cost of real-time PCR instruments has been decreasing in recent years. However, they still remain expensive for many research laboratories. Real-time PCR systems, such as those available from commercial companies, often include all the hardware required to perform the assay, including the thermal cycler, computer, fluorescence optics, and software. However, to perform DEF-PCR, the hardware could be limited, in theory, to a thermocycler and fluorimeter.

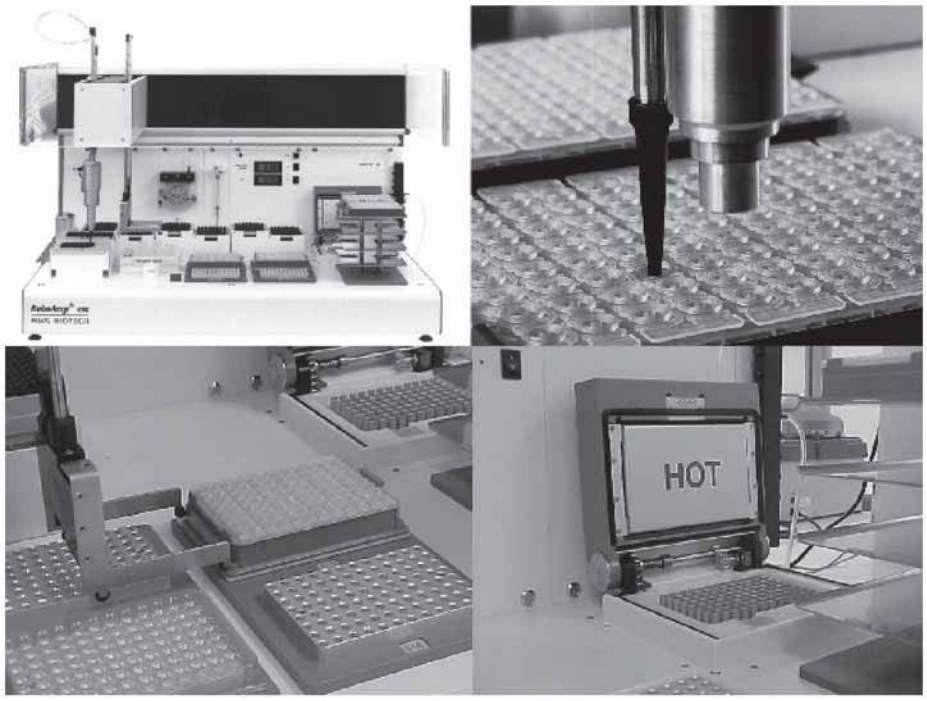

Fig. 1. A typical robotic liquid handling system showing the robot, liquid handling system, microtiter plate holder, and thermocycler (clockwise from top left).

As an example of DEF-PCR, the detection of three bacterial pathogens is used, namely *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemphilus influenzae*. These bacteria are important causes of meningitis and septicemia worldwide ***(21–24)***. All three cause sporadic disease, but *N. meningitidis* also can cause outbreaks ***(25,26)***. The rapid progression of symptoms and potentially devastating effect of this disease necessitate early recognition and immediate treatment ***(27)***. It is therefore essential to be able to detect and characterize these bacteria when they cause disease.

2. Materials

The assumption is made that the DEF-PCR method is performed in a high-throughput and fully automated setting (*see* **Fig. 1**). The hardware described is therefore more than may be required in some settings but these can be scaled back according to individual needs (*see* **Notes 1–3**).

1. Roboseq 4204 SE robotic liquid handling system possessing an integrated vacuum manifold, thermocycler, and fluorescence reader (such as Bio-Tek FL600 fluorescence plate reader with KC4 software; MWG Biotech).

Table 1
Oligonucleotide Primers and Probes Used in DEF-PCR for the Detection of *N. meningitidis*, *S. pneumoniae,* and *H. influenzae*

Name	Label 5′	Label 3′	Organism	Sequence (5′-3′)
Oligonucleotide primers				
ctrAF			*N. meningitidis*	GCT GCG GTA GGT GGT TCA A
ctrAR			*N. meningitidis*	TTG TCG CGG ATT TGC AAC TA
plyAF			*S. pneumoniae*	TGC AGA GCG TCC TTT GGT CTA T
plyAR			*S. pneumoniae*	CTC TTA CTC GTG GTTTCC AAC TTG A
bexAF			*H. influenzae*	GGC GAA ATG GTG CTG GTA A
bexAR			*H. influenzae*	GGC CAA GAG ATA CTC ATA GAA CGT T
Dual-labeled probes				
CtrA	FAM	TAMRA	*N. meningitidis*	CAT TGC CAC GTG TCA GCT GCA CAT
BxA	TET	TAMRA	*S. pneumoniae*	CAC CAC TCA TCA AAC GAA TGA GCG TGG
PlyA	HEX	TAMRA	*H. influenzae*	TGG CGC CCA TAA GCA ACA CTC GAA

2. Appropriate filters for selected wavelengths of 485/530 nm, 530/590 nm, and 590/645 nm are required to detect the fluorescence emissions.
3. Genomic DNA extraction kit, such as the Promega Wizard SV 96 System (Promega), for the isolation of genomic DNA from whole blood, serum, and plasma.
4. ABsolute™ QPCR Mastermix (ABgene): Thermostart Taq DNA polymerase, dNTPs, magnesium chloride, reference dye (ROX), and reaction buffer. PCR mastermix can be stored at 4°C for as long as 1 mo but should otherwise be stored at temperatures less than 0°C, preferably at –20°C, and protected from light.
5. Repeated freeze–thawing should be avoided (*see* **Note 4**).
6. 1.8-mL Non-crosscontamination (NCC) tubes (Web Scientific, Crewe, UK).
7. Optically clear disposable strips (ABgene).
8. 96-Well microtiter plate.
9. Oligonucleotide primers (1 pmol; MWG Biotech). These primers were based on previously published *ctrA*, *ply*, and *bexA* gene sequences for meningococcal, pneumococcal, and *H. influenzae* DNA, respectively (*see* **Table 1 *[13,28]***). The *bexA* probe is supplied by Biosource International (Camarillo, CA). Oligonucleotide primers should be stored at 4°C for as long as 1 wk or at –20°C for as long as approx 3 mo.
10. Dual-labeled probe (0.5 pmol; MWG Biotech). Probes should be stored at –20°C in the dark for approx 3 mo.
11. 200-mL Filter pipet tips.
12. Sterile distilled water.

3. Methods

Bacterial DNA is extracted from clinical samples using a liquid handling robot and automated DNA binding plate system. The robotic system performs all liquid handling, thereby allowing the rapid extraction of as many as 96 samples. This produces a high yield of bacterial DNA for use in PCR, with a theoretical sensitivity of one to two genome copies per 100-µL sample and specificity of 100% for each organism. The PCR set-up and reaction also is automated on the liquid-handling robot. The robot is programmed, according to the manufacturer's instructions, to perform as many as 96 samples in one run, allowing high throughput with good accuracy and minimal manual intervention. The use of a robot also reduces the possibility of contamination because the robot has a NCC system.

3.1. Sample Requirements

For positive and negative controls, grow bacterial cells under the optimal conditions for the organism to be detected, either on solid or in liquid media. For *N. meningitidis* and *S. pneumoniae*, this is usually on Colombia blood agar with horse blood and, for *H. influenzae*, usually on chocolate blood agar, overnight at 37×C in 5% CO_2. Appropriate media should be used for these organisms if grown in liquid media, such as brain heart infusion broth. If grown on solid media, emulsify approx 12 colonies in 100 µL of sterile distilled water. If using liquid media, take 100 µL of well-mixed culture. If using clinical samples, an aliquot of 100 µL of fluid is required.

3.2. Extraction of Genomic DNA

1. Using an aliquot of 50 µL of sample, one should largely follow the manufacturer's instructions for extracting genomic DNA using the Promega Wizard SV 96 System, but modifications can be made according to the requirements for automation (*see* **Note 5**).
2. Add 100 µL of bacterial culture or body fluid to 200 µL of SV RNA lysis buffer and vortex to mix.
3. Program the robot to automatically place the SV96 DNA binding plate on top of the vacuum manifold.
4. Add 150 µL of blood lysate to the wells of the binding plate.
5. Apply a vacuum of 600 millibars for approx 2 min or until the solution has passed through the membrane.
6. Add 1 mL of SV96 Wash solution to each well and apply a vacuum as before for approx 2 min.
7. Repeat the washing step once.
8. Again apply a vacuum at 600 millibars for 5 min to remove residual ethanol.
9. Place a collection plate inside the vacuum manifold and add 75 µL of nuclease-free water to each well on the binding plate.
10. Apply a vacuum of 600 millibars for 1 min to elute the DNA into a 1.8-mL NCC tube.

3.2. Fluorescence-Based PCR

1. Program the robot to set up PCR in a final volume of 25 µL consisting of 20 µL of ABsolute™ QPCR Mastermix, 1 µL of each primer (1 pmol final concentration), 1 µL of probe (0.5 p*M* final concentration), and 2 µL of DNA.
2. PCR amplification is performed using the cycle, 95°C for 2 min, followed by 45 cycles of 95°C for 15 s, 53°C for 30 s, and 72°C for 2 min followed by a final extension at 72°C for 3 min.
3. To determine the assay cut-off value for negative and positive samples, five negative controls and one of each positive control are used. Negative controls consist of PCR mix without target DNA, but include primers, and the final volume made to 25 µL with sterile distilled water. The positive control consists of PCR mix, 1 µL of each primer, and 2 µL of control DNA.
4. Wavelengths of 485/530 nm, 530/590 nm, and 590/645 nm are used to determine the fluorescence produced by the CtrA, PlyA, and BexA probes, respectively. A total of 100 endpoint readings are taken from each well. The average reading is determined by using the KC4 software and a cut-off value calculated using 1.4 standard deviations above the mean fluoresence for the five negative controls.

4. Notes

1. The method is relatively inexpensive compared with similar methods because the chemistry is widely available without the need for specialist equipment and, as such, can be performed manually using conventional set-up techniques and results read with a manually operated fluorescent plate reader possessing the appropriate filter set.
2. Liquid handling robots are available from various commercial manufacturers. The actual system set-up often can be modified according to individual requirements such that various assays can be performed on the same platform.
3. The methodology described assumes that the user has been fully trained on robot programming or the manufacturer has provided preprogrammed methods as requested by the user. The specific tasks performed by the robot can be modified as necessary.
4. PCR mastermix can be stored at 4°C for as long as 1 mo for ready access if reagents are used regularly. However, this storage is not recommended for occasional use and, instead reagents should preferably be stored at –20°C. They also should be protected from light. Repeated freeze–thawing should be avoided. All reagents should be maintained at 4°C while on the robotic platform. Failure to do so can result in reduced sensitivity and specificity of assays.
5. Genomic DNA extraction kits can be purchased commercially for manual or automated methods. A study compared a number of different methods to determine the most suitable for automation by determining ease of use, sensitivity, and specificity among 96-well binding plate, 96-well filter plate, and metallic bead formats ***(19)***. Not all kits are specifically designed for all DNA extraction requirements, but most can be modified and validated accordingly.

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6

Use of Hybridization Probes in a Real-Time PCR Assay on the LightCycler® for the Detection of Methicillin-Resistant *Staphylococcus aureus*

Andrea J. Grisold and Harald H. Kessler

Summary

The rapid and accurate identification of methicillin-resistant *Staphylococcus aureus* (MRSA) is of great importance for the affected patient, the involved ward, and the microbiological laboratory. Resistance to methicillin is encoded by the *mecA* gene in *S. aureus*. Because routine laboratory diagnostics may be time consuming and because species differentiation encounters a variety of difficulties, molecular techniques detecting both the *mecA* and a *S. aureus*-specific gene are used for rapid and accurate detection and identification of MRSA. Various protocols, including the manual extraction of DNA have been established. In this chapter, the identification of MRSA based on simultaneous detection of the *mecA* gene and the *S. aureus*-specific Sa442 DNA fragment using automated DNA extraction and real-time polymerase chain reaction is described. This method is an attractive alternative to labor-intensive manual protocols and can easily be incorporated into the diagnostic microbiology laboratory workflow, with the ability to obtain results within 4 h.

Key Words: RSA; Real-time PCR; methicllin; mecA; sa442; LightCycler® Instrument; *Staphylococcus aureus*.

1. Introduction

Staphylococcus aureus is one of the most significant human pathogens producing nosocomial and community-acquired infections. The wide spectrum of clinical manifestations include superficial, deep-skin, and soft-tissue infections, osteomyelitis, pneumonia, endocarditis, and septicemia as well as a variety of toxin-mediated diseases, including food-borne gastroenteritis, staphylococcal-scalded skin syndrome, and toxic shock syndrome, with β-lactam antibiotics being the drugs of choice for therapy ***(1)***.

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Since the introduction of semisynthetic penicillins (such as methicillin and, subsequently, oxacillin) into clinical use in the 1960s, the occurrence of methicillin-resistant *S. aureus* (MRSA) strains has increased steadily and mainly nosocomial infections caused by such isolates have become a serious problem worldwide, with varying degrees of prevalence ***(2,3)***.

Methicillin resistance in *S. aureus* is caused by the acquisition of the exogenous *mecA* gene. This gene encodes for an additional penicillin-binding protein, referred to as PBP2a (or PBP2), with a low binding affinity for β-lactam antibiotics and consequently resistance of these strains to all β-lactam antibiotics. In MRSA, however, additional resistance to other classes of antibiotics often is observed. In rare cases, resistance to methicillin is induced by the hyperproduction of β-lactamases or the production of methicillinases, resulting in borderline resistance ***(4,5)***.

The evidence of MRSA has important implications for the treatment and management of the patient. Glycopeptides or oxazolidinones remain the drugs of choice for antibiotic therapy. However, strains with intermediate susceptibility to vancomycin have been isolated ***(6,7)***.

In the clinical laboratory, *S. aureus* is identified by growth characteristics followed by detection of catalase and coagulase activities. Conventional susceptibility testing of *S. aureus* detects resistance to oxacillin and other classes of antibiotics by agar dilution tests, disk diffusion tests, or agar screening methods according to the standards of the National Committee of Clinical Laboratory Standards (NCCLS ***[8]***). *S. aureus* is usually easy to identify by conventional culture techniques. However, because resistance to oxacillin in staphylococci is heterogenous and sometimes difficult to induce, accurate determination of oxacillin-resistance in staphylococci may be time consuming, requiring 2 to 4 d. Heterogeneity implies differences in inoculum size, incubation time, medium pH, and medium salt concentration ***(9–11)***. False-negative or even noninterpretable results may be observed when commercially available kits for coagulase testing are used ***(12,13)***.

Today, the rapid and sensitive detection of the *mecA* gene by nucleic acid amplification has evolved as the method of choice for rapid and accurate identification of MRSA ***(14–16)***. Molecular methods for the rapid identification of MRSA are based on the detection of the *mecA* and a *S. aureus*-specific gene target, for instance, the Sa442 deoxyribonucleic acid (DNA) fragment ***(17–19)***. The Sa442 DNA fragment, which is a popular DNA target for identification of *S. aureus* by polymerase chain reaction (PCR) methods, originally was described by Martineau et al. ***(20)***. If DNA extraction is performed on an automated device followed by combined amplification and sequence specific detection of amplication products by real-time PCR, a total assay time less than 4 h can be achieved. It must, however, be taken into consideration that molecular

detection of MRSA does not include determination of resistance to other classes than β-lactam antibiotics.

2. Materials

2.1. General Equipment

1. Turbidimeter (Densicheck).
2. MagNA Pure LC instrument or MagNa Pure Compact (Roche; *see* **Note 1**).
3. LC Caroussel Centrifuge (Roche).
4. LightCycler® instrument (Roche; *see* **Note 2**).
5. LightCycler software (*see* **Note 2**).
6. Heating block.

2.2. Reagents

1. Amplification primers.
2. Hybridization probes.
3. Lysozyme.
4. 10 m*M* Tris-HCl, pH 8.0.
5. Phosphate-buffered saline (PBS).
6. PCR-grade water.
7. MagNA Pure LC DNA Isolation Kit III for use on the MagNA Pure LC instrument (Roche) or MagNa Pure Compact Nucleic Acid Isolation Kit I for use on the MagNA Pure Compact (Roche).
8. LightCycler Fast Start DNA Master Hybridization Probes (Roche).

3. Methods

3.1. Bacterial Isolation and Preparation of Suspension

1. Culture bacteria on blood or any other nutrient agar for staphylococci according to the NCCLS recommendations.
2. Suspend staphylococcal strains in 2 to 3 mL of PBS buffer, with a turbidity of 0.5 McFarland corresponding to 1.5×10^8 bacteria per milliliter.

3.2. Preparation of Lysozyme Solution

Take lysozyme and prepare a solution of 10 mg/mL in 10 m*M* Tris-HCl, pH 8.0. Aliquots may be kept at –20° C for long-term storage.

3.3. Sample Preparation

1. The MagNA Pure LC DNA Isolation Kit III or the MagNa Pure Compact Nucleic Acid Isolation Kit I for DNA extraction may be used.
2. External lysis: When using the MagNA Pure LC DNA Isolation Kit III, add 100 µL of the bacterial suspension to a mixture of 130 µL Bacterial Lysis Buffer and 20 µL of proteinase K-solution in a 1.5-mL tube (according to the manufacturer's package insert). Add an additional 5 µL of lysozyme solution and incubate for 10

min at 65°C, followed by 10 min at 95°C. When using the MagNA Pure Compact Nucleic Acid Isolation Kit I, add 5 µL of lysozyme solution to 100 µL of the bacterial suspension in a 2.0-mL tube (included in the kit) and incubate for 10 min at 65°C followed by 10 min at 95°C.

3. DNA extraction: When using the MagNA Pure LC DNA Isolation Kit III, transfer 200 µL of the lysate into a well of the sample cartridge and start the automated DNA extraction protocol on the MagNA Pure LC instrument (standard protocol; *see* **Note 1**). After completion of the automated DNA extraction procedure, the eluted DNA (elution volume 100 µL) is ready for the postelution protocol (*see* **Notes 3** and **4**). When using the MagNA Pure Compact Nucleic Acid Isolation Kit I, put the 2.0-mL tube containing the lysate into the designated position on the MagNa Pure Compact. Start the automated DNA extraction protocol (blood protocol; *see* **Note 1**). After completion of the automated DNA extraction procedure, the eluted DNA (elution volume 100 µL) is ready for the manual preparation of the PCR mixes (*see* **Notes 3** and **4**).

3.4. Real-Time PCR

3.4.1. Primers and Probes

Oligonucleotide primers and fluorescence-labeled hybridization probes are designed for amplification and sequence-specific detection of both a 188-bp fragment within the *mecA* gene and a 178-bp fragment within the *S. aureus*-specific Sa442 gene (*see* **Note 5**). Nucleotide sequences and positions are listed in **Table 1.** Lyophilized primers are diluted in PCR-grade water (*see* **Note 6**). Concentrations of the stock solution are listed in **Table 2**.

3.4.2. Master Mix

1. Place the required number of LightCycler Capillaries (number of samples and controls) in a precooled sample carousel.
2. Prepare the master mix by multiplying the component volume per capillary by the number of capillaries, plus one additional (excess volume for pipetting).
3. When using the MagNA Pure LC instrument, place the MagNA Pure LC cooling block, including the sample carousel with the adequate number of LightCycler capillaries and the reaction vessels (master mix, negative control, positive control) into the postelution area of the MagNA Pure LC instrument. After the start of the postelution protocol, which has to be programmed prior to the start of the run, the MagNA Pure LC instrument automatically pipets 18 µL of the master mix and 2 µL of the eluted sample into each of the LightCycler capillaries. After finishing the postelution protocol, take out the cooling block and carefully cap the capillaries. When using the MagNA Pure Compact, take the cooling block, including the sample carousel with the adequate number of LightCycler capillaries and the reaction vessels (master mix, negative control, positive control), and prepare PCR mixes manually (18 µL of the master mix and 2 µL of the eluted sample respectively controls into each of the LightCycler capillaries). Cap the capillaries carefully.

Table 1
Oligonucleotide Primers and LightCycler Hybridization Probes Used for the PCR Assay

Oligonucleotide	5´-3´ Sequence [a]	Target gene	Nucleotide positions	GenBank accession no.	Refs.
Sa442-F	GTCGGGTACACGATATTCTTCACG	*Sa442*	12–34	AF033191	***17***
Sa442-RS	CTCGTATGACCAGCTTCGGT	*Sa442*	189–168	AF033191	***17***
Sa442-HP-1	TACTGAAATCTCATTACGTTGCATCGGAA-[FAM]	*Sa442*	95–123	AF033191	***18***
Sa442-HP-2	[Red 705]-ATTGTGTTCTGTATGTAAAAGCCGTCTTG-[Ph]	*Sa442*	126–154	AF033191	***18***
Mec-S	CTAGGTGTGGTGAAGATATACCA	*mecA*	1596–1619	X52592	***17***
Mec-A	TGAGGTGCGTTAATATTGCCA	*mecA*	1783–1763	X52592	***17***
Mec-HP-1	CAGGTTACGGACAAGGTGAAATACTGATT-[FAM]	*mecA*	1690–1718	X52592	***18***
Mec-HP-2	[Red 640]-ACCCAGTACAGATCCTTTCAATCTATAGCG-[Ph]	*mecA*	1720–1739	X52592	***18***

[a] [FAM], fluorescein; [Red 705], LightCycler-Red 705-phosphoramidite; [Ph], 3´-phosphate, [Red 640]-LightCycler-Red-640-N-hydroxysuccinimide ester.

Table 2
Master Mix for Amplification and Hybridization Probe-Based Detection of the *mecA*- and *S. aureus*-Specific Amplification Products

Components	Volume per capillary (µL)	Final
LightCycler- DNA Master Hybridization Probes	2	1X
$MgCl_2$ stock solution (25 m*M*)	3.2	5 m*M*
Primers mecA (50 µ*M* each)	0.4 + 0.4	1 µ*M*
Primers Sa442 (5 µ*M* each)	0.3 + 0.3	0.75 µ*M*
Hybridization probes MecA (20 µ*M* each)	0.2 + 0.2	0.2 µ*M*
Hybridization probes sa442 (20 µ*M* each)	0.2 + 0.2	0.2 µ*M*
H_2O (PCR grade)	18	

Centrifuge the LC Carousel at 3000 rpm for 1 min in the specially designed LC Carousel Centrifuge.

4. Place the Carousel into the LightCycler and start the run.

3.4.3. LightCycler® PCR Protocol

The following PCR protocol is used for amplification and hybridization probe-based detection of the *mecA* gene and the *S. aureus*-specific Sa442 DNA fragment. The protocol consists of the following steps:

1. Denaturation: 1 cycle of 10 min at 95°C
2. Amplification: 50 cycles as shown in the following table:

Parameter	**Value**		
Cycles	50		
Analysis mode	Quantification		
	Segment 1	Segment 2	Segment 3
Target temperature [°C]	97	50	72
Hold time [s]	10	10	15
Temperature transition rate- slope [°C/s]	20	20	20
Acquisition mode	None	Single	None

3. Melting curve analysis: 1 cycle as shown in the table below

Parameter	**Value**		
Cycles	1		
Analysis mode	Melting Curve		
	Segment 1	Segment 2	Segment 3
Target temperature [°C]	95	40	85
Hold time [s]	60	120	0
Temperature transition rate- slope [°C/s]	20	20	0.2
Acquisition mode	None	None	Step

4. After the final cycle, the capillaries must be cooled for 2 min at 40°C.

3.5. Data Analysis

Fluorescence curves can be analyzed with the LightCycler software (*see* **Note 2**). Automated calculation of the crossing points must be done by the second derivative maximum method. The fluorescence of each capillary can be measured at wavelengths of 640 and 705 nm (dual-color option). Select cycles from 0 to 50 and channel 640 for the *mecA* gene and channel 705 for the *S. aureus*-specific gene (Sa442). Each result has a specific peak in the corresponding melting curve (*see* **Fig. 1**).

3.6. Suggested Controls

3.6.1. Positive Controls

Oxacillin-susceptible *S. aureus* strains such as *S. aureus* ATCC (American Type Culture Collection, Manassas, VA) 29213, ATCC 25923, or NCTC (National Collection of Type Cultures and Pathogenic Fungi, Colindale, UK) 8325 can be used, in addition to MRSA strain NCTC 10442 or MRSA ATCC 33591.

3.6.2. Negative Controls

Negatives controls include 5 µL of distilled water and *Enterococcus faecalis* (ATCC 29212).

4. Notes

1. Both of the instruments are automated, closed laboratory benchtop devices for isolation of nucleic acids. Contamination risk is minimized. The MagNA Pure LC instrument allows automated nucleic acid extraction of, at maximum, 32 specimens. The MagNA Pure Compact allows, at maximum, 8 specimens.
2. All currently available LightCycler versions and corresponding software programs may be used.
3. It is advisable to look for any discrepancies, for example, a plugged tip or clots in the samples after completion of DNA extraction. When using the MagNA Pure LC instrument, the liquid discard button should never be turned on. The aforementioned problems will not be noticed then because the instrument will discard everything in the waste container.
4. Use extracted DNA immediately or close the wells of the sample with cartridge seals if using the MagNA Pure LC instrument or close the tubes MagNA Pure Compact and store the extracted sample at +2 to +8°C for, at maximum, 24 h or at –20°C for long term.
5. A recent work describes rare cases with Sa442-negative *S. aureus* as a result of a sequence variation in the primer binding site(s) or a deletion of (part of) the corresponding gene from the genome ***(21)***.
6. Because variations in lot-to-lot primer concentrations may exist, it is advisable to adjust the concentrations for each primer lot prior to its first use in routine diagnostics.

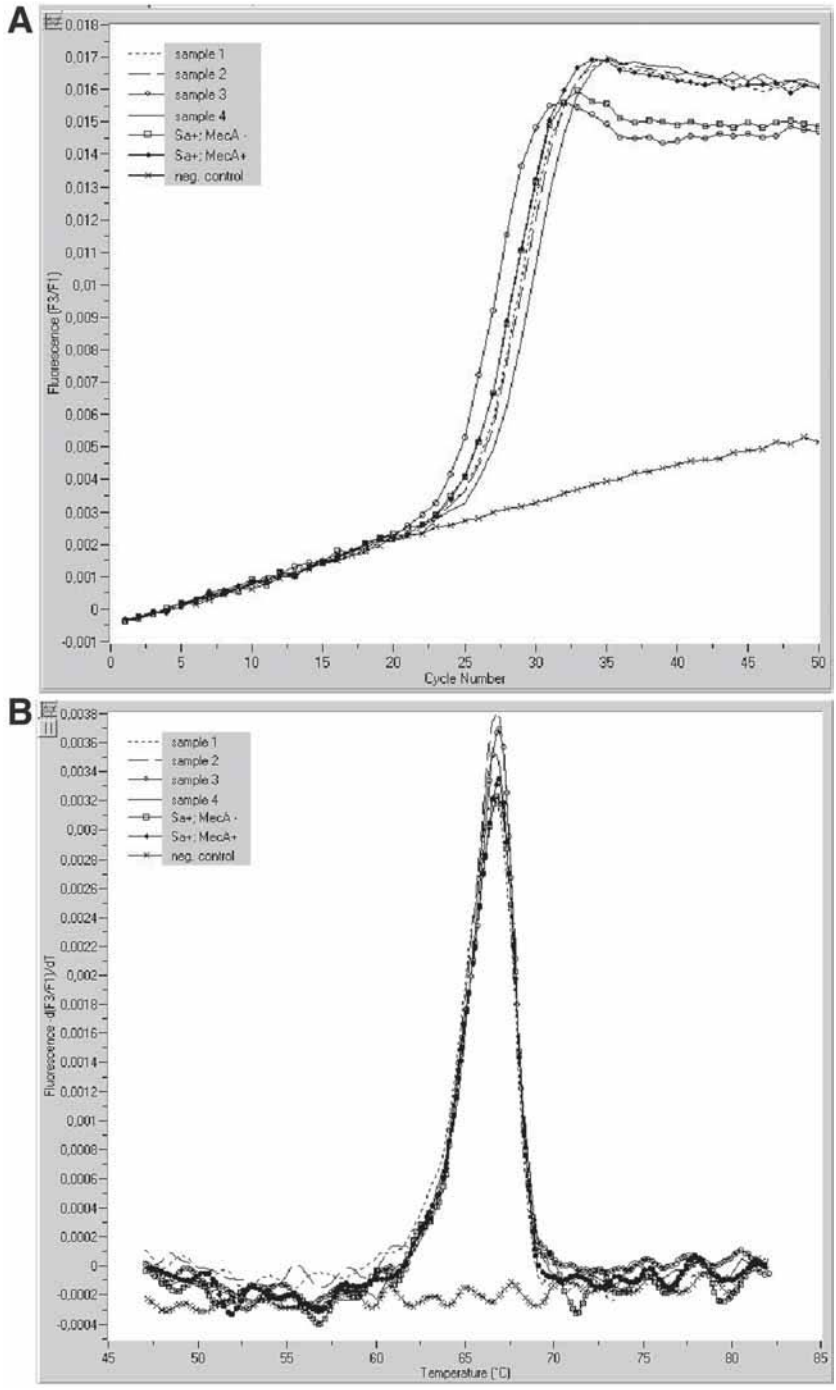
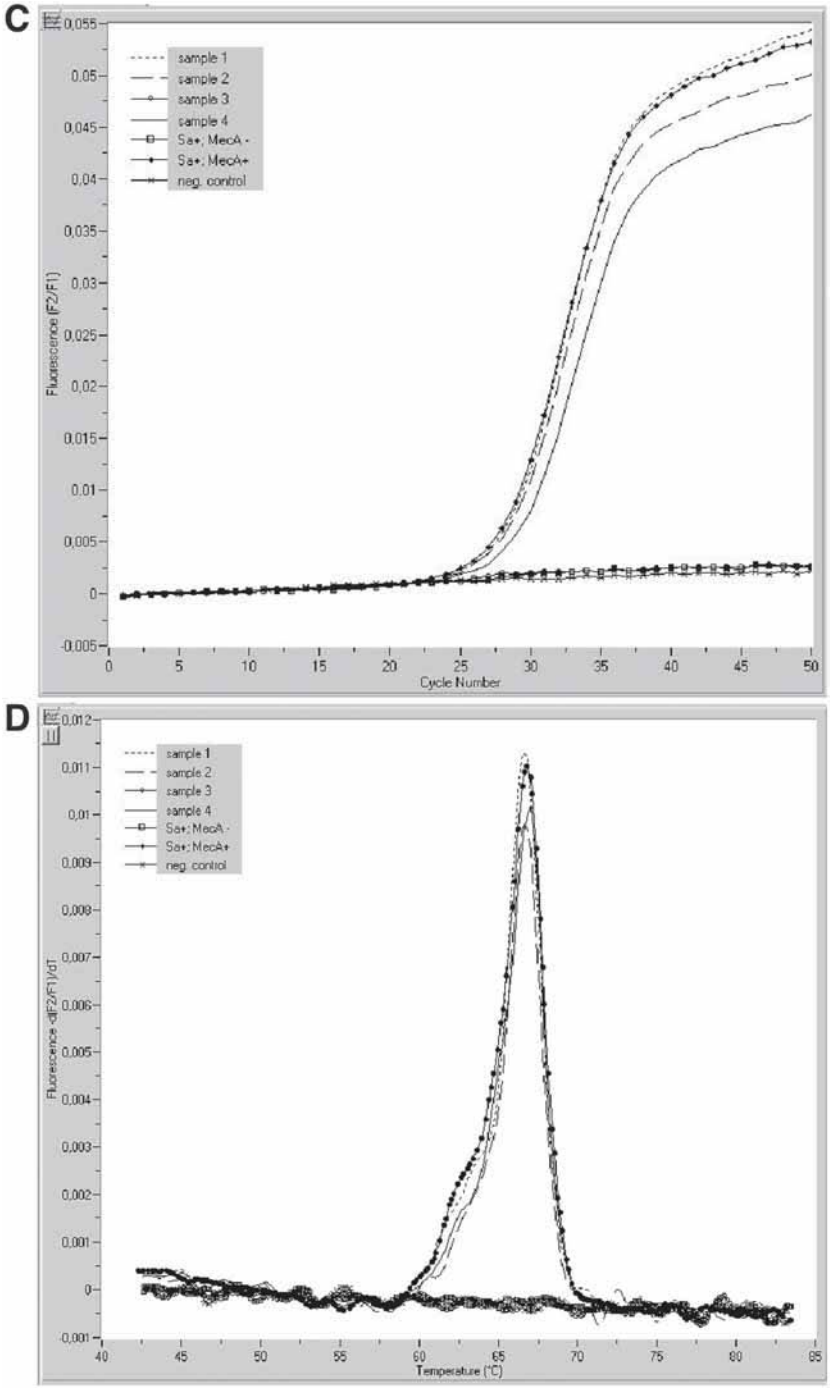


Fig. 1. Quantification curves (fluorescence vs cycle number plots) for *S. aureus*-specific genomic fragment Sa442 of clinical samples **(A)** and the corresponding melting curves, which shows identical product peaks for all samples **(B)**, quantification curves for



mecA gene of clinical samples **(C)**, and the corresponding melting curves **(D)**. Probes for Sa442 are labeled with LC dye 640 and those for *mecA* gene are labeled with LC dye 705, allowing the convenient identification of both products.

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7

Detection of Verotoxin Genes *VT 1* and *VT 2* in *Escherichia coli* O157:H7 in Minced Beef Using Immunocapture and Real-Time PCR

Justine Fitzmaurice

Summary

Escherichia coli O157:H7 is a highly virulent pathogen that causes severe food poisoning in humans. Many outbreaks involving this pathogen have been linked to minced beef. A protocol is presented here to detect *E. coli* O157:H7 in minced beef. The method consists of an enrichment step in modified tryptone soya broth, followed by immunomagnetic separation and extraction of deoxyribonucleic acid. Real-time polymearse chain reaction, using hybridization probes, is used to detect the verotoxin genes 1 and 2 found in *E. coli* O157:H7. The assay has a detection limit of $\log_{10}$ 3.5/mL *E. coli* O157:H7 cells in minced beef.

Key Words: Real-time PCR; *Escherichia coli* O157:H7; verotoxin genes; *VT 1*; *VT 2*; diagnostics.

1. Introduction

Escherichia coli O157:H7 is a highly virulent food poisoning pathogen capable of causing severe gastrointestinal illness in humans and is the most well-known member of the group of *E. coli* called verocytotoxigenic *E. coli* (VTEC). VTEC are characterized by the production of verotoxins, which are termed verotoxin 1 (VT 1), verotoxin 2 (VT 2), and several subtypes of VT 2 also have been identified.

Detection of *E. coli* O157:H7 in food presents a number of challenges. The pathogen may be present in very small numbers, may be stressed or injured, and may be present with high numbers of competing bacteria. In addition, the food matrix itself, consisting of proteins, fats, oils, or other compounds, may

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inhibit the recovery of the pathogen of interest. As a result, there is a need for diagnostic assays to overcome or minimize the effect of these problems.

This chapter describes a method to detect the verotoxin genes *VT 1* and *VT 2*, found in *E. coli* O157:H7, in minced beef. This protocol begins with enrichment of minced beef in a selective broth for 18 h. Enrichment of the sample allows the number of the target bacteria to be increased while decreasing the number of nontarget or competing bacteria ***(1,2)***. Immunomagnetic separation (IMS) is used to recover the pathogen from the enriched minced beef. IMS uses magnetic beads coated with an antibody that is specific for the *E. coli* O157 antigen. The beads are allowed to bind to the target cells in suspension and the bead–cell complex is subsequently removed by the application of a magnetic field ***(2–5)***. This process allows both the isolation of *E. coli* O157 from nontarget bacteria and concentration of the pathogen ***(4)***. DNA is extracted from the IMS beads by using a phenol:chloroform-based method. Because DNA extraction does not depend on the bacteria of interest being in a particular physiological state, DNA also will be extracted from stressed or injured cells. The *VT 1* and *VT 2* genes are identified by a real-time PCR assay, consisting of one set of primers designed to amplify both *VT 1* and *VT 2* genes, and specific hybridization probes are used to distinguish the two verotoxin genes.

2. Materials

2.1. Enrichment of Minced Beef

1. Modified tryptone soya broth (mTSB).
2. 0.02 g/L Novobiocin.
3. Stomacher®400 Circulator (Seward Lab Systems).
4 Stomacher bags (Seward Lab Systems).

2.2. IMS

1. IMS beads specific for *E. coli* O157–*E. coli* O157-IMS SEIKEN beads (Denka Seiken).
2. 0.9% Sterile saline.
3. IMS Rotating Mixer-MMV14 (Heto-Holten).

2.3. Extraction of DNA

1. Lysis buffer: 2% Triton X-100, 1% sodium dodecyl sulfate, 100 m*M* NaCl, 10 m*M* Tris-HCl, pH 8.0, 1 m*M* ethylene diamine tetraacetic acid (*see* **Note 1**).
2. Phenol:chloroform:isoamyalcohol (25:24:1).
3. 3 *M* Sodium acetate.
4. Absolute ethanol.
5. 70% Ethanol.
6. Nuclease-free water.

Table 1
PCR Primers and Hybridization Probes

Name	Sequence (5´-3´)	T_m (°C)
Consensus F primer	GGCARATACAGAGRGRATTTCGT	51.4
Consensus R primer	ATGYCAATTCAGTATWAKMGCCAC	52.8
VT 1 FLU probe	CCTGCCTGACTATCATGGACAAGACTCT	63.6
VT 1 LC640 probe	TTCGTGTAGGAAGAATTTCTTTTGGAAGC	63.8
VT 2 FLU probe	TACTCCGGAAGCACATTGCTGAATC	62.8
VT 2 LC705 probe	CCCCCAGTTCAGAGTGAGGTCC	63.4

2.4. Real-Time PCR

1. LightCycler® real-time PCR machine (Roche).
2. LightCycler DNA master hybridization probes kit (Roche).
3. LightCycler capillaries (Roche).
4. 0.5 µ*M* Consensus F and consensus R primers (*see* **Table 1**).
5. 0.2 µ*M* *VT 1* FLU, *VT 2* FLU probes (*see* **Table 1**).
6. 0.4 µ*M* *VT 1* LC, *VT 2* LC probes (*see* **Table 1**).
7. Nuclease-free water.
8. LightCycler Color Compensation Set (Roche).

3. Methods

3.1. Enrichment of Minced Beef

1. Place 25 g of minced beef in a sterile filter bag with 225 mL of mTSB + novobiocin and stomach for 2 min.
2. Incubate at 41.5°C, without shaking, for 18 h.

3.2. IMS

1. Remove 1 mL from the enriched minced beef sample and place in a sterile 1.5-mL microcentrifuge tube (*see* **Note 2**).
2. Using the dropper provided add one drop of IMS beads to the sample and rotate in a rotating mixer for 30 min at room temperature.
3. Place the microcentrifuge tube in a magnetic rack and rotate by hand until the beads are visibly drawn to the side of the tube.
4. With the magnet still in place, remove and discard the supernatant.
5. Remove the tube from the magnetic rack and add 1 mL of 0.9% sterile saline. Mix by inversion three times to wash.
6. Place the tube in the magnetic rack and rotate until the beads are drawn to the side of the tube. Remove and discard the supernatant.
7. Repeat the washing step.
8. Resuspend the beads in 100 µL of 0.9% sterile saline.

3.3. Extraction of DNA

1. Add a 50-µL aliquot of resuspended IMS beads, with captured *E. coli* O157 cells to 500 µL of lysis buffer and 200 µL of phenol:chloroform:isoamyalcohol in a 1.5-mL microcentrifuge tube. Vortex for 30 s.
2. Centrifuge at 10,600*g* for 5 min.
3. Remove 400 µL of the resulting supernatant and add to 40 µL of 3 *M* sodium acetate and 800 µL of absolute ethanol in a 1.5-mL microcentrifuge tube. Mix by inversion three times and incubate at –20°C for a minimum of 30 min (*see* **Note 3**).
4. Centrifuge at 20,800*g* for 15 min. Remove and discard the supernatant. Resuspend the pellet in 200 µL of 70% ethanol.
5. Centrifuge at 20,800*g* for 5 min, remove supernatant and allow pellet to air dry (*see* **Note 4**).
6. Resuspend pellet in 75 µL of nuclease-free water.

3.4. Real-Time PCR

1. Prepare a master mix per reaction as follows (*see* **Note 5**):

10X LightCycler DNA master hybridization probes buffer	2 µL (1X)
25 m*M* $MgCl_2$	3.2 µL (4 m*M*)
10 pmol/µL consensus F primer	1 µL (0.5 µ*M*)
10 pmol/µL consensus R primer	1 µL (0.5 µ*M*)
4 pmol/µL *VT 1* FLU probe	1 µL (0.2 µ*M*)
8 pmol/µL *VT 1* LC probe	1 µL (0.4 µ*M*)
4 pmol/µL *VT 2* FLU probe	1 µL (0.2 µ*M*)
8 pmol/µL *VT 2* LC probe	1 µL (0.4 µ*M*)
Nuclease-free H_2O	6.8 µL

2. Dispense 18-µL aliquots of the master mix into glass capillaries.
3. Add 2 µL of DNA template to each capillary. Include a no-template control in each run, replace template with 2 µL of nuclease-free H_2O. Seal each capillary with a stopper.
4. Spin down the capillaries in the LightCycler carousel centrifuge (*see* **Notes 6** and **7**).
5. Run the cycling conditions as follows: initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 5 s, annealing at 53°C for 30 s, and extenstion at 72°C for 10 s.
6. Analyze data using the LighyCycler software (*see* **Notes 8** and **9**). **Figure 1** shows typical data.

4. Notes

1. Use molecular grade reagents for preparation of all solutions.
2. Ensure no fat is included when removing an aliquot of enriched minced beef as this will inhibit the binding of the IMS beads.
3. During the DNA extraction procedure, it is possible to lengthen the incubation at –20°C to overnight or longer. It is a convenient stopping point in the procedure.

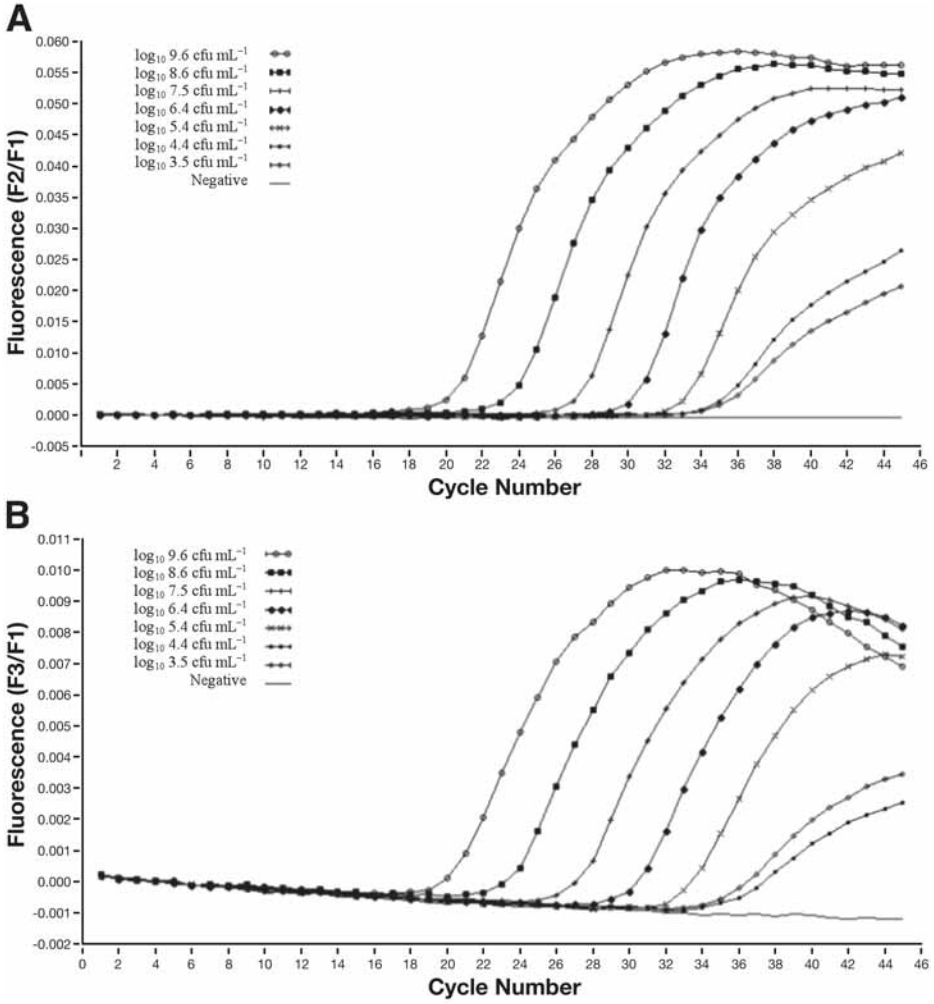

Fig. 1. Real-time polymerase chain reaction sensitivity data using deoxyribonucleic acid extracted from cells captured from enriched minced beef samples by immunomagnetic separation. **(A)** shows the *VT 1*-specific signal in the F2/F1 channel and **(B)** shows the *VT 2*-specific signal in the F3/F1 channel.

4. Ensure all ethanol is evaporated from tubes before resuspending the DNA in H_2O.
5. The 10X LightCycler DNA master hybridization probes buffer requires storage between 2 and 8°C after thawing. All other components of the real-time PCR assay should be stored at –20°C in small volumes of working stocks to avoid repeated freezing and thawing. Because the fluorescent labels on the probes are sensitive to light, it is recommended that one prepare new dilutions of probe for each reaction and to not expose them to fluorescent light for long periods.

6. Wear gloves when handling the capillaries and avoid touching the surface of the capillaries as this may affect the fluorescent readings taken by the LightCycler.
7. Precool the capillaries before dispensing the master mix.
8. It is advisable to generate a color compensation file on the LightCycler using the LightCycler color compensation set from Roche. This file is a requirement to perform dual-color assays. It is used to compensate for crosstalk between channels when both LightCycler Red 640- and LightCycler Red 705-labeled probes are used in the same reaction. It is recommended that a new color compensation file is created every 6 mo.
9. The detection limit of the method presented here has been determined to be $\log_{10}$ 3.5/mL *E. coli* O157:H7 cells in minced beef. This is based on three independent experiments, using spiked enriched minced beef and derived serial dilutions ranging from $\log_{10}$ 9.6/mL to $\log_{10}$ 3.5/mL.

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8

Application of Two-Step Quantitative Reverse-Transcription PCR to Bacterial Diagnostics

Barry Glynn

Summary

Use of high copy number bacterial RNA offers several advantages in a diagnostics context compared with current deoxyribonucleic acid-based assays. The opportunity to only detect viable cells by targeting labile RNA transcripts may create an opportunity for "real-time" monitoring of pathogen load in response to a treatment regimen, while the natural amplification provided by the relative abundance of the RNA target compared with its corresponding gene opens a door to potential nonamplified direct detection technologies. In this chapter, a method is described to accurately quantify specific RNA transcripts and thus determine their potential utility as "high-copy" targets. The quantification method described also has application in gene-expression analysis.

Key Words: RT-PCR; in vitro-transcribed standards; cRNA; real-time PCR.

1. Introduction

Polymerase chain reaction (PCR)-based diagnostic assays are well established in the food, environmental, and clinical microbiology sectors ***(1)***. The advent of real-time PCR has heralded an upturn in the speed and specificity of these assays ***(2)***. Paradoxically, the high sensitivity of this technology has potentially become an obstacle to its applicability in the clinical setting, especially where residual amounts of bacterial DNA persisting in a patient sample are present ***(3)***. The potential of RNA-based assays to circumvent this obstacle and only detect live cells has been described ***(4,5)***. High copy number bacterial RNAs have been investigated for their ability to identify bacterial species ***(6)***, and a useful first step in these analyzes is the determination of transcript copy number for potential RNA targets. Quantification of specific RNA copy number in a clinical, environmental, or food sample also may provide a means to rapidly monitor the

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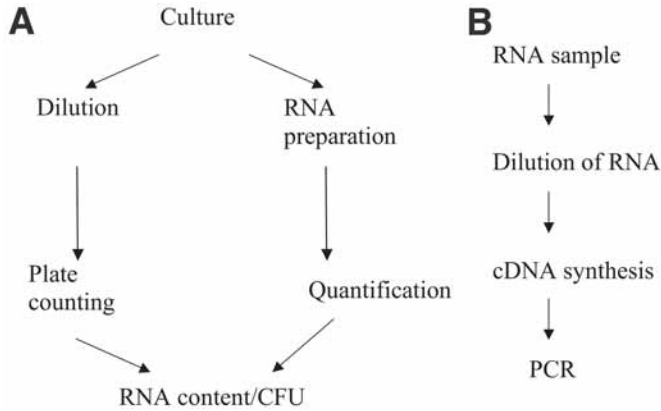

Fig. 1. Workflow of quantification experiment. **(A)** Calculation of recoverable RNA content per CFU. This calculation is useful if the intention is to normalize copy number to CFU. **(B)** Steps involved in quantitative reverse-transcriptase PCR from both standard curves and total RNA (unknowns)

number of viable bacteria present in the sample. The application of an RNA quantification methodology as part of a theranostic strategy to monitor bacterial load during an antibacterial treatment also may provide useful information that cannot currently be obtained by either measurement of deoxyribonucleic acid (DNA) levels in a sample or by more time consuming conventional microbiological approaches ***(7)***.

Quantification experiments **(Fig. 1)** typically are described as being one of three types, absolute, relative, and comparatitive. Despite optimistic claims to the contrary, all quantification experiments are "relative" only approaching the grail of being "absolute" as the quality of the standards improves. To provide an accurate reflection of in vivo copy number, an in vitro system must be devised to mirror the intracellular state or, as a compromise, to allow the conditions used for enumeration of 'unknowns" be replicated in the preparation of the standards.

Real-time PCR using 5´ exonuclease probe technology particularly is suited to the quantification of gene copy number ***(8–10)***. An intrinsic characteristic of real-time PCR technology is that the cycle at which the fluorescent signal crosses the detection threshold (Ct value) is proportional to the starting concentration of target material. Standard curves may be prepared using known amounts of synthetically generated target material ***(2,11)***. For estimation of RNA copy number in samples, an in vitro transcribed complementary RNA (cRNA) standard homologous to the transcript being quantified is recommended for the generation of the standard curve ***(9,12)***.

Table 1
Comparison of DNA and RNA Standard Curves

Template	CT value
1×10^7 PCR products	22.25
1×10^7 RNA products	31.48
1×10^6 PCR products	25.73
1×10^6 RNA products	34.50
1×10^5 PCR product	29.10
1×10^5 RNA products	36.00

There is a difference in amplification efficiency between RNA (RT-PCR) and DNA (PCR) templates in a quantitative reaction. As expected, purified PCR products are a much more efficient amplification template than RNA and, as such, should not be used in RNA quantification experiments. A dilution series of quantified PCR products has an amplification efficiency of $e = 1.959$, whereas a dilution series of the same amount of RNA templates has an efficiency of $e = 2.777$. The Ct values returned from these templates also differ to such an extent that a quantification experiment based on the PCR products as standards would result in a 500-fold (nine-cycle Ct difference) under estimation of the RNA copy number.

In a quantitative reverse-transcriptase (qRT)-PCR as with any quantitative analysis an essential element is the use of a "like-for-like" comparison between standard curves and the "unknown" material being analyzed. Because the materials being quantified in these experiments are RNA transcripts, the first step in this like-for-like analysis is the preparation of a set of artificially generated RNA transcripts for use in the standard curves (*see* **Note 1**). These transcripts are designed to be identical in sequence to the transcript being quantified. Therefore, a separate standard must be prepared for each RNA that is being quantified. A second level of complexity in the like-for-like analysis of standards and unknowns involves comparison of the amplification efficiencies of the various templates (*see* **Note 2**). Analysis of the mathematical models available for both RT-PCRs and PCRs demonstrate that even a slight fluctuation in reaction efficiencies can result in major differences in calculated number of initial RNA molecules (*see* **Table 1**).

To give an accurate representation of target copy number, the amplification efficiency of the standard curve should be as close as possible to the amplification efficiency of the unknowns. For example, if the amplification efficiency of the standard curve is high whereas there is some inhibition of amplification

in the unknowns (resulting in a lower amplification efficiency), the quantification data returned will not be an accurate reflection of the target copy number. The standard curve depends in part upon the amplification efficiency of the standards. Therefore, every effort should be made to ensure that the amplification efficiencies of the standard curve and the unknowns are identical. Amplification efficiency (*e*) is calculated from the slope of a graph of the Ct values of a dilution series (either standards or unknowns) using the equation $e = 10^{-1/S}$, where *S* = slope ***(13)***.

In this chapter, a protocol is described for the quantification of high copy number RNAs in bacteria using in vitro transcribed cRNA standards and two-step qRT-PCR on the LightCycler® instrument.

2. Materials

The quantification methodology described consists of four sections, in vitro transcription of cRNA for use in standard curves, preparation of unknowns (including determination of total RNA content per cell), two-step qRT-PCR protocols, and data analysis (including measurement of amplification efficiencies).

2.1. Preparation of cRNA for Standard Curves

1. Conventional thermocycler.
2. *Taq* polymerase and 10X PCR buffers (Gibco-BRL).
3. dNTPs (Promega) .
4. Heating blocks.
5. Invitrogen TOPO® TA Cloning Kit.
6. 37°C incubators (Shaking and plate incubators).
7. Water bath.
8. Gel electrophoresis apparatus.
9. Ambion MAXIscript™ in vitro transcription kit.
10. Ambion NUCaway™ columns.
11. Agilent 2100 bioanalyzer and RNA 6000 Nano LabChip® kit.
12. Ambion RNA 6000™ ladder.

2.2. Preparation of Unknowns

1. Cell density meter.
2. RNA preparation (Ambion Ribopure Yeast™ kit is recommended or the Roche High-Pure viral RNA purification kit).

2.3. Two-Step qRT-PCR

1. Conventional thermocycler.
2. 100 U/µL M-MLV reverse transcriptase (Ambion).
3. 10X Reaction buffer: 500 m*M* Tris-HCl, pH 8.3, 500 m*M* KCl, 30 m*M* $MgCl_2$, 50 m*M* dithiothreitol (Ambion).
4. dNTPs (Promega).

5. QIAquick PCR Product Purification Kit (Qiagen).
6. Real-time PCR machine (LightCycler was used for these experiments).

2.4. Data Analysis

We use the integrated LightCycler data analysis software.

3. Methods

3.1. Preparation of cRNA for Standard Curves

The ultimate goal of the experimental design is to create a set of standards that mimic, as closely as possible, the conditions of the unknown sample being quantified. Therefore, in the case of an RNA copy number experiment, it is useful to use RNA in the preparation of the standard curve. At an early stage, the use of in vitro-transcribed RNA in the standard curve instead of quantified PCR products replicates the RT step carried out on the unknown samples (*see* **Note 3**).

Preparation of RNA standards involves the following steps:

- Amplification by conventional PCR of target gene.
- Ligation into suitable plasmid vector.
- Transformation into competent cells.
- Plasmid purification.
- Screening to identify insert orientation relative to promoter site.
- In vitro transcription.
- Treatment with DNase.
- RNA purification and quantification.
- Dilution of RNA template to suitable concentration.

3.1.1. Amplification of the Target Gene

PCR amplification of the gene being quantified is performed using standard conditions. In the examples shown here a short (~200) bp fragment of the 16s ribosomal RNA (rRNA) gene from *L. monocytogenes* was amplified.

1. Prepare PCR mix for gene amplification in a 50-µL volume. The reaction mix should consist of 200 n*M* of each primer, 400 µ*M* dNTP, 5 µL 10X reaction buffer, and 1 U *Taq* polymerase.
2. Amplify the target DNA in a conventional thermocycler. In the case of 16S rRNA, amplification conditions are 1 cycle of 95°C for 5 min followed by 30 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 45 s, and a final extension step of 72°C for 5 min.

3.1.2. Ligation Into Plasmid Vector

pCR® II.1 TOPO® cloning kit can be used for preparation of suitable clones. This plasmid has both T7 and Sp6 RNA polymerase promoter sites suitable for

the in vitro transcription of specific RNA from the cloned insert (*see* **Note 4**). Follow the manufacturer's instructions for use.

3.1.3. Transformation Into Competent Cells

1. Transform 1 µL of the ligation reaction into *E. coli* Inf' competent cells (they are included in the invitrogen kit) and incubate overnight on Luria-Bertani (LB) plates containing ampicillin and X-gal.
2. After an overnight incubation on LB agar plates containing X-gal, select eight white colonies and inoculate into 5 mL LB broths containing antibiotic.
3. Incubate overnight at 37°C with shaking.

3.1.4. Plasmid Purification

1. Prepare plasmid DNA from the 5-mL overnight incubations using QIAprep® Spin Miniprep Kit.
2. After purification, digest a 5-µL aliquot with *Eco*R1 at 37°C for 1 h.
3. Run the entire restriction reaction on 1.4% agarose gel.
4. Before proceeding to in vitro transcription, it is necessary to identify the orientation of the insert in the plasmid vector. To generate a sense RNA transcript, it is necessary for the 5´ end of the RNA to lie adjacent to the RNA polymerase promoter site. Sequencing of the cloned plasmid generates unambiguous data on the insert orientation as well as adding a confirmation that the correct gene was cloned.

3.1.5. In Vitro Transcription

To ensure that the cRNA produced by in vitro transcription is of a defined length, plasmids should be linearised using restriction enzyme *Hin*dIII (*see* **Note 5**).

1. Incubate plasmid in 50-µL volume at 37°C for 1 h.
2. Purify the plasmid using QIAquick® PCR product purification kit and run a 10-µL aliquot on 1.5% agarose gel to confirm complete digestion.
3. Conducte in vitro transcription using 1 µg of plasmid DNA as template with the MAXIscript™ in vitro transcription kit for 1 h at 37°C.

3.1.6. Treatment With DNase

After incubation, DNase treat the samples using the DNase 1 *DNAfree*™ (*see* **Note 6**).

3.1.7. Purification and Quantification of RNA

1. Remove free nucleotides, that is, dNTPs and rNTPs, by gel filtration over Nuc-Away™ Spin Columns.
2. Quantify 1-µL aliquots of the RNA on the Agilent 2100 bioanalyzer with RNA 6000 Nano LabChips®.
3. Store RNA at –20°C until use in the RT-PCR.

3.1.8. Dilution of RNA Template to a Suitable Concentration

Prepare 10-fold serial dilutions of DNase-treated RNA to span the range of concentrations from 1 ng/µL to 10 fg/µL (*see* **Note 7**).

3.2. Preparation of Unknowns

Preparation of unknowns for quantification involves the following steps:

- Determination of total RNA content/cell.
- Total RNA preparation from appropriate growth phase.
- Quantification of RNA.
- Treatment with DNase.
- Dilution of RNA to appropriate concentration (typically one cell equivalent).

3.2.1. Determination of Total RNA Content/Cell

1. Prepare a standard curve of bacterial growth to identify stationary and exponential phases of growth.
2. Grow cultures to appropriate growth phase (typically mid-exponential phase and stationary phase after 24 h).
3. Prepare a 10-fold serial dilutions of culture by adding 20 to 180 µL of sterile broth. Dilutions should be prepared in triplicate (*see* **Note 8**).
4. Prepare appropriate dilutions and spread onto labeled agar plates. Incubate overnight at 37°C. Remove duplicate 5-mL aliquots from the culture and prepare total RNA using High-Pure viral RNA purification kit. After purification, quantify the RNA by measuring triplicate 5-µL aliquots of each preparation in a 500-µL volume at 260 nm in an ultra violet spectrophotometer.
5. Calculate the mean RNA content of the six measurements and extrapolate to the total RNA extracted from the 5 mL of culture.
6. After the overnight incubation, count the colonies on the plates.
7. Calculate the mean of the triplicate samples and extrapolate the CFU/mL of culture using the dilution factor. Calculate the mean RNA content/cell by dividing the total RNA per milliliter of culture by the total CFU/mL (*see* **Note 9**).

3.2.2. Preparation of Total RNA

1. Incubate cultures for appropriate length of time to reach mid exponential and stationary phases of growth.
2. Remove duplicate 5-mL aliquots from cultures and pellet bacteria by centrifugation.
3. Discard the supernatant and resuspend cells in 200 µL buffer before applying to Ribopure Yeast™ kit (recommended) or to High Pure Nucleic Acid purification columns.

3.2.3. Quantification of RNA

1. Duplicate 1-µL aliquots of total and in vitro-transcribed RNA should be quantified on the Agilent 2100 Bioannalyzer and RNA 6000 Nano LabChip® system.
2. Alternatively, RNA may be quantified using optical density readings at 260 nm or by the use of RNA-specific dyes.

3.2.4. Treatment With DNase

Treat samples with DNase using the *DNAfree*™ protocol.

3.2.5. Dilution of RNA to Appropriate Concentrations

1. Prepare 10-fold serial dilutions by adding 2 µL of RNA to 18 µL of RNase-free water.
2. Serial dilutions from containing RNA equivalents of 10 to 1000 cells should be used to overcome potential PCR inhibitor effect of reverse transcriptase at low template concentration (*see* **Note 10** ***[13]***).

3.3. Two-Step qRT-PCR

3.3.1. RT

1. Incubate 1 µL of each dilution at 80°C for 3 min with 100 ng of reverse primer in 12 µL of volume.
2. Quench on ice before adding reaction mix containing 1 µL of M-MLV Reverse Transcriptase (100 U/µL), 2 µL of 10X reaction buffer, and 4 µL of 2 m*M* dNTP mix.
3. Conduct reverse transcription at 42°C for 1 h.
4. Denature RNA/DNA hybrids and RT enzyme at 95°C for 10 min.
5. Store cDNAs at –20°C.

3.3.2. Real-Time PCR of cDNA

PCR amplification and real time detection was performed on the LightCycler instrument with TaqMan® probes.

1. Prepare 20-µL reaction volumes for each sample using amplification conditions optimized for the gene transcript being quantified.
2. Templates should include standard curve cDNAs to span the range of expected target transcripts. Typical standard curves should contain dilutions containing from 1×10^3 to 1×10^7 copies.
3. Templates from total RNA should include cDNA prepared from a dilution series corresponding to from 10 to 1×10^3 cell equivalents.

3.4. Data Analysis

For quantification analysis, design standard curves to span the expected range of RNA copy numbers. Include in each experiment dilutions corresponding to RNA copy number from 1×10^3 to 1×10^7. Reverse transcribe unknown

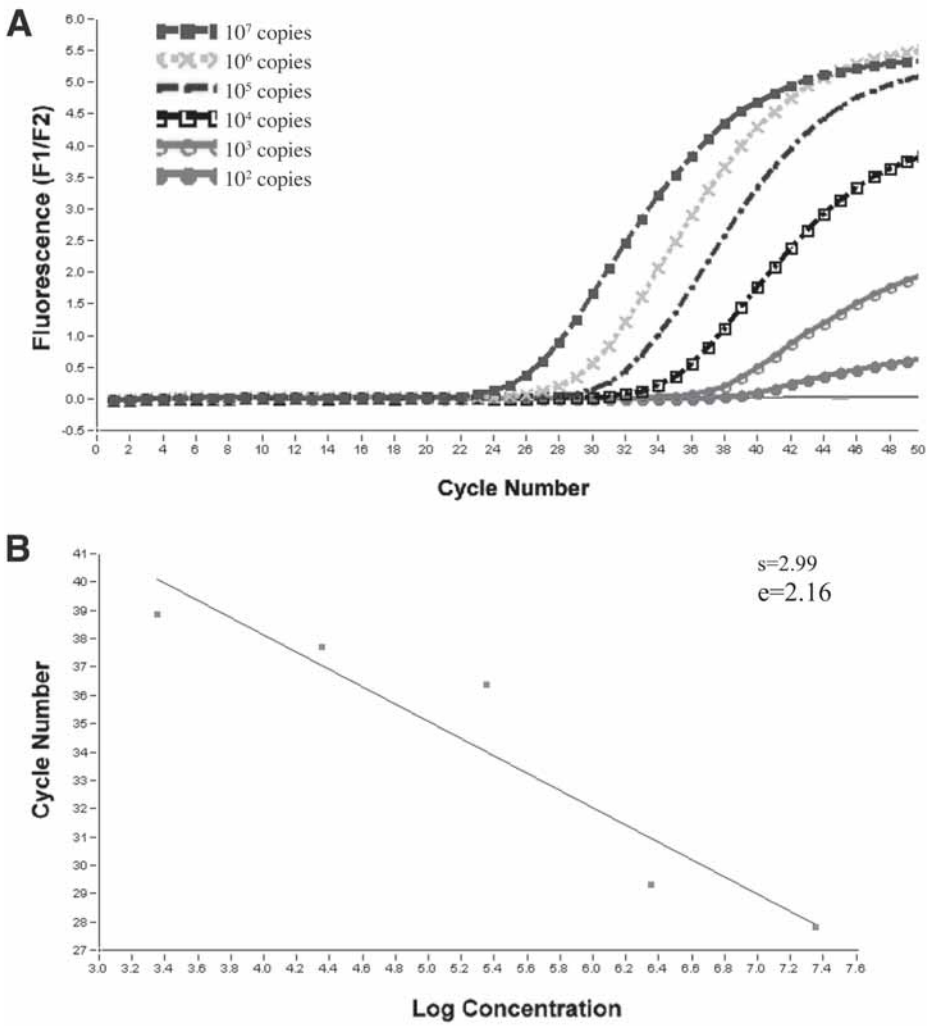

Fig. 2. Measurement of amplification efficiencies of standard curves and unknowns. Shown in **(A)** are amplification curves from a serial dilution of in vitro-transcribed RNA templates. **(B)** shows the plot of the Ct values of the standard curve dilution series and the associated slope (s) and reaction efficiency (e). *(Continued on next page)*

RNA corresponding to known cell equivalents and amplify by real-time PCR in parallel with the standards.

1. Integrated LightCycler data analysis software is used to plot a graph of the Ct values of both standard curve and dilutions of total RNA.
2. The slope of this line(s) can be used to calculate the amplification efficiency of the reactions (e) by application of the equation $e = 10^{-1/S}$ *(see* **Fig. 2***)*.

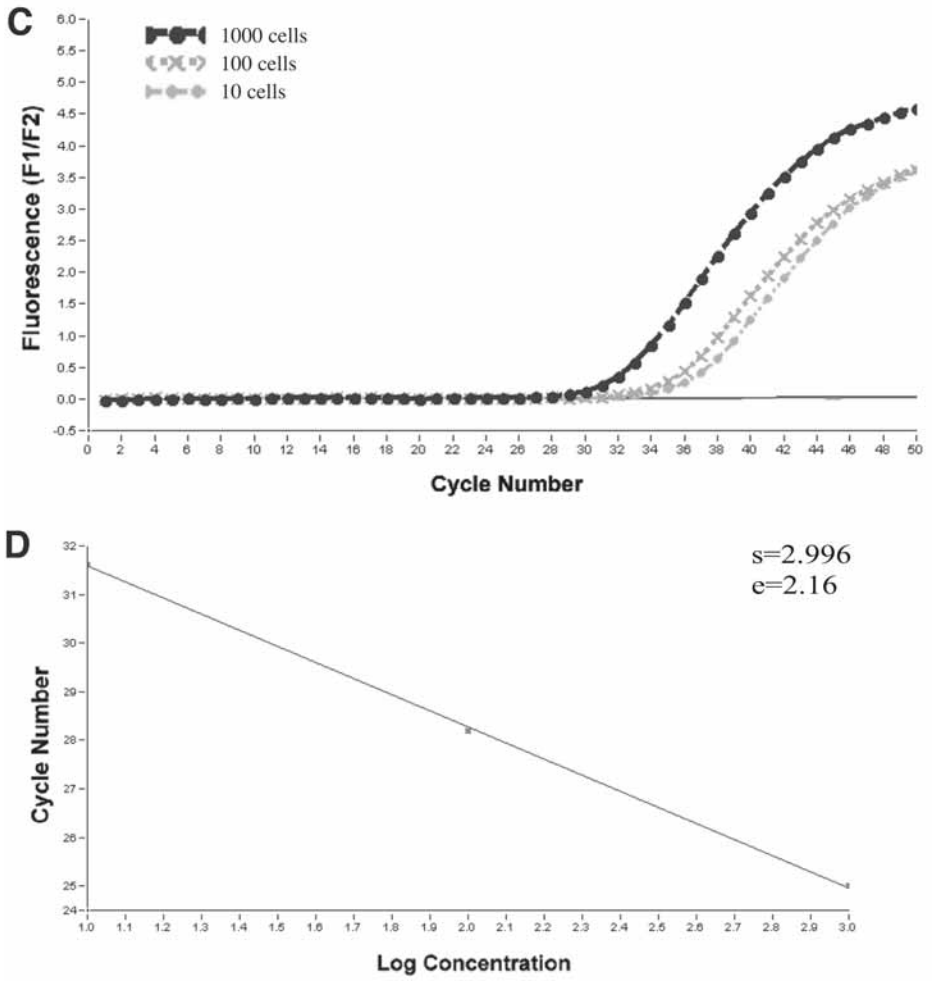

Fig. 2. *(Continued from previous page)* **(C)** and **(D)** illustrate the amplification curves and plot of a dilution series prepared from total RNA. Amplification efficiencies of both sets of templates are equivalent, meaning that interpolation of copy number from these sets of amplifications can be performed in confidence.

3. Amplification efficiencies of standards and unknowns must be as close as possible for accurate quantification data to be returned.

4. Notes

1. RNA standards are prepared by in vitro transcription from a T7 promoter that has been attached to the 5´ end of the target gene. This generates a homogenous population of RNA molecules of known length. This material is quantified and the number molecules/microliter calculated. Serial dilutions of this RNA provide the template for the generation of an RT-PCR standard curve.
2. Any RT-PCR experiment that uses dilutions of RNA as starting material also will be influenced by inhibition of the RT reaction. The alternative approach, preparing a large stock of cDNA and diluting down from, this will not display this problem. There may be inhibition in the single large cDNA reaction but, because the standard curve is not designed to measure RT efficiency, just the subsequent PCR, its effect remains unnoticed. Amplification efficiencies are calculated by measuring the slope of a plot of Ct values for a dilution series of template ***(7)***, $e = 10^{-1/S}$.
3. Short (~200 bp) PCR products from the genes of interest are cloned into plasmids containing a T7 RNA polymerase promoter site. In vitro transcription is used to generate RNA transcripts consisting of the cloned fragment and a short plasmid sequence. These transcripts are then column purified and quantified by ultraviolet spectroscopy. One 200-base RNA transcript is calculated to be equivalent to 0.2 ag (1 ag = 1×10^{-18} g) of this RNA. Serial dilutions of the in vitro-transcribed RNA are used as template for RT, and this cDNA is amplified using the LightCycler.
4. An alternative approach to generate starting template for in vitro transcription involves using PCR where the forward primer has been modified to include the T7 promoter sequence (taatacgactcactatagg). We chose the cloning strategy described here because it generates a stable pool of material from which fresh template can be prepared.
5. T7 RNA polymerase is highly processive and will continue transcription until a stop signal is encountered. In the case of a circular plasmid, the polymerase could potentially continue indefinitely. The restriction digestion prevents this and also results in the RNA transcripts being of uniform known size, which eases quantification calculations.
6. Several methodologies suggest purification of the in vitro-transcribed RNA immediately after transcription. However, it is our experience that it is advisable to perform DNase treatment of the prepared RNA *before* column purification. This step not only reduces the overall number of purification steps by one (it is no longer necessary to conduct a separate post-DNase purification) but it also facilitates the column purification of both free nucleotides and ribonucleotides from the mix in one step. Column purification after DNase treatment also has been shown to improve the accuracy of spectrographic readings taken to quantify nucleic acid concentration.
7. Several online calculators are available to determine the molecular weight of an RNA transcript. A 200-base RNA will have an average molecular weight of 64 kDa, which corresponds to a single 200-base transcript having a mass of 0.1ag or 1×10^{-19}g

8. Because of the large numbers of plates that will be involved, it is recommended that an initial "ranging" experiment be carried out to identify which dilutions will result in countable plates. Prepare 10-fold dilutions and plate 200 mL onto plate. Incubate overnight at 37°C. Examine the plates to identify which of the dilutions result in between 30 and 300 CFU/plate. These are the dilutions that should be used in the triplicate plate count experiment.
9. The figures generated for amount of RNA per cell are limited by several factors. First, the CFU per milliliter may underestimate the total number of cells present in the original culture as not all cell produce a colony. Second, no RNA preparation method can be guaranteed to result in 100% purification of RNA from cultures. Therefore, we apply the term "recoverable RNA yield CFU^{-1}" to more accurately describe our starting material.
10. At low concentrations of template, RT inhibits subsequent PCRs. The inhibitorary effect of RT on PCR is removed at template concentrations beyond 10^5 copies ***(14)***. When designing standard curves, it is useful to avoid lower dilutions of template because the amplification efficiency calculations will be affected by this inhibitory effect. An alternative solution mentioned (*see* **Note 11**) also may be useful.
11. We also have investigated the possibility of using a dilution series of cDNA samples to generate the standard curves. Provided that the amplification efficiencies of these samples match those prepared from the RNA dilution series, this method provides several advantages. First, the total number of RT reactions is reduced; second, the cDNA template used to prepare the standards is more stable than an in vitro-transcribed RNA; third, this method is more accurate at lower template dilutions because it eliminates an inhibitory effect of components of the RT reaction on the PCR amplification. We found that diluting the high copy number cDNA gave more consistent standard curves and a lower limit of detection (down to 100 copies) while having a similar amplification efficiency as the more laborious RNA dilution method.

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9

Quencher Extension for Single Nucleotide Polymorphism Quantification in Bacterial Typing and Microbial Community Analyses

Knut Rudi, Monika Zimonja, and Beate Skånseng

Summary

Quencher extension is a novel single-step closed tube real-time method to quantify single nucleotide polymorphisms (SNPs) in combination with primer extension. A probe with a 5´-reporter is single-base extended with a dideoxy nucleotide containing a quencher if the target SNP allele is present. The reaction is measured from the quenching (reduced fluorescence) of the reporter. The relative amount of a specific SNP allele is determined from the nucleotide incorporation rate in a thermocycling reaction. The quencher extension protocol presented was developed for SNP allele quantification in *Listeria monocytogenes* and for microbial community analyses.

Key Words: Single nucleotide polymorphism; SNP; multilocus sequence typing; MLST; *Listeria monocytogenes*; microbial communities; small subunit ribosomal RNA gene; 16S rDNA; real-time; reporter/quencher assay.

1. Introduction

Most bacteria cannot be grown or analyzed in pure culture. Therefore, a need exists for analyzing bacteria in mixed populations. Extensive DNA sequence information about bacterial diversity is now accumulating in public databases such as GenBank (http://www.ncbi.nlm.nih.gov/) and the EMBL Nucleotide Sequence Database (http://www.ebi.ac.uk/embl/). Therefore, many single nucleotide polymorphism (SNP) alleles for discriminating bacteria are known. The challenge, however, is to use this information for analyses of bacteria in mixed populations ***(1)***.

There are a lack of proper tools to quantify SNP alleles (differences between bacteria caused by single-point mutations ***[2]***). We have therefore developed

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quencher extension (QEXT). QEXT is a novel technique for the quantification of SNP alleles ***(3)***. The principle of QEXT is that a probe with a reporter dye is single-base extended with a dideoxy nucleotide containing a quencher if the target SNP allele is present. The signal is recorded from the quenching (reduced fluorescence) of the reporter dye (*see* **Fig. 1**). QEXT is directly adaptable to most of the current real-time polymerase chain reaction (PCR) equipment that is available.

The QEXT protocol presented is derived from analyses of SNP alleles in both *Listeria monocytogenes* populations ***(3)*** and from microbial community analyses of chicken cecal samples. SNP alleles in the *inlA* gene were used for *L. monocytogenes*, while 16S rDNA was used for the microbial community analyses ***(4)***.

L. monocytogenes is an important human pathogen with a mortality rate of approx 30% ***(5)***. The problem with this bacterium is that it is very abundant in the environment and that most of the *L. monocytogenes* strains actually are nonpathogenic to humans. Thus, it is very important with rapid-screening techniques to determine the presence of the virulent *L. monocytogenes* types. Recently, multilocus sequence typing (MLST) has emerged as a typing technique ***(6)***. The disadvantage of this technique is that DNA sequencing is a labor-intensive process. Furthermore, most of the information generated by MLST is not informative because it is only SNP alleles (polymorph sites) that can be used for diagnostics. With the accumulating MLST knowledge, however, SNP alleles can be identified that are diagnostic for the virulent *L. monocytogenes* strains ***(7)***. QEXT has proven very useful for rapid screening of SNP alleles in *L. monocytogenes* ***(3)***. Similar approaches also should be easily adaptable to MLST data for other bacteria.

A major challenge with microbial community analyses is the quantification of several different bacteria in complex samples. We have recently developed DNA array approaches for the semiquantitative description of microbial communities ***(1)***. The limitation, however, is the throughput of DNA array approaches when analyzing relatively few probes. In these cases, it is simpler and more accurate to use QEXT.

2. Materials

In addition to standard laboratory equipment, the QEXT analyses require real-time PCR instrumentation. The protocol described here was developed using the ABI PRISM 7900 HT instrument (Applied Biosystems; *see* **Note 1**).

2.1. Template

Any PCR-amplification product can be used as template in the QEXT reaction. We recommend using 1 μ*M* of each PCR primer in the amplification reaction to ensure that enough targets are present for the QEXT reaction.

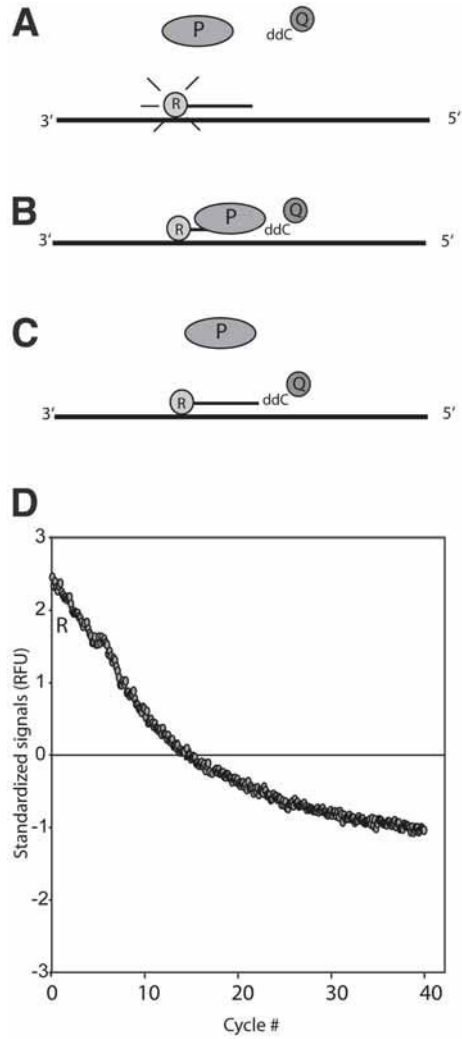

Fig. 1. Schematic representation of the QEXT method. DNA is shown as black bars, the DNA polymerase as ovals (P), the dideoxy cytosine labeled with a quencher as ddC with a circle (Q), and the 6-carboxyfluorescein reporter as a circle with (R). **(A)** Before incorporation of the acceptor, R emits fluorescence. **(B)** A DNA polymerase incorporates the quencher containing dideoxy cytosine if the target SNP allele is present. **(C)** After sequence-specific incorporation of the quencher, the emitted fluorescence from R is quenched. **(D)** Standardized multicomponent signals (by subtracting the mean and dividing on the standard deviation) for R relative to labeling cycles. The curve decreases proportional to the incorporation rate of the dideoxy cytosine per cycle. The curve is exponential because the incorporation rate is dependent on the incorporation from the previous cycles.

2.2. QEXT Probes

1. QEXT probes prelabeled at the 5´-end with 6-carboxyfluorescein (FAM) with the 3´-ends unmodified (*see* **Note 2**).

2.3. Pretreatment of PCR Products

1. 2 U/µL Shrimp alkaline phosphatase (USB).
2. Glycine buffer, pH 10.4, at 37°C.
3. 10 U/µL Exonuclease I from calf intestine.

2.4. QEXT Reaction

1. 32 U/µL Modified thermostable DNA polymerase Thermo Sequenase (Amersham Biosciences).
2. *Thermoplasma acidodophilum* inorganic pyrophosphatase.
3. Thermo Sequenase Reaction Buffer: 260 m*M* Tris-HCl, pH 9.5, 65 m*M* $MgCl_2$ (Amersham Biosciences; *see* **Note 3**).
4. Dideoxy cytosine labeled with 6-carboxy-*N*,*N*,*N´*,*N´*-tetramethylrhodamine (TAMRA; *see* **Note 4**).
5. MicroAmp Optical Adhesive Covers and 96-well Optical Reaction Plates (Applied Biosystems).
6. Microtiter plate centrifuge.

2.5. Data Analysis

We provide a Microsoft Excel (Microsoft) macro for analyses of the analyses of the of the multicomponent output data from the Applied Biosystems 7900 HT instrument. The macro will run on any ordinary personal computers with Microsoft Excel 97 or newer versions. The macro can be downloaded from www.matforsk.no/webprosj/qext.nsf.

3. Methods

The QEXT protocol has three general steps. First, there is a sample pretreatment, removing the residual nucleotides and PCR primers from the template to be used. The next step is the actual QEXT reaction, and the final step is the analysis of the output data.

3.1. Pretreatment of PCR Products

The following protocol is used to dephosphorylate the nucleotides and to remove single-stranded DNA.

1. Transfer 10 µL of PCR product to a fresh PCR tube.
2. Add 0.5 µL (1 U) of shrimp alkaline phosphatase and 0.5 µL (5 U) of exonuclease I.
3. Incubate at 37°C for 1 h in a thermocycler and then inactivate the enzymes by heating to 95°C for 10 min.
4. Store the products at 5°C before use in the QEXT reaction.

3.2. QEXT Reaction

All components except the template should be mixed in master mix prior to adding them to the wells in the Optical Reaction Plates. The volumes and concentrations are given per well in the reaction plate.

The reaction mix is prepared by combining the following reagents in a tube as follows.

1. 0.6 µL of Concentrated Thermo Sequenase Reaction Buffer.
2. 0.1 µL of TAMRA-labeled dideoxynucleotides (100 µ*M*).
3. 0.2 µL of QEXT probe stock solution 10 µ*M* (*see* **Note 5**).
4. 0.1 µL of Thermo Sequenase (32 U/µL).
5. 2 µL of exonuclease I and shrimp alkaline phosphatase treated PCR product.
6. Add water to a final reaction volume of 10 µL.
7. The reaction mix is added to each well in the 96-well reaction plate, and the plate is sealed with MicroAmp Optical Adhesive Covers and then centrifuged at 110*g* for 1 min.
8. The 96-well reaction plate is then placed in the 9700 HT instrument.
9. The following settings are used for running the machine. Choose:
   a. Assay: absolute quantification (Standard curve);
   b. Container: 96-well clear plate; and
   c. Template: blank template.
10. Select add detector and use a detector with FAM reporter and TAMRA quencher.
11. Choose no passive reference.
12. Go to instrument settings and modify the thermocycling conditions as follows

    Stage 1: Use an initial denaturation at 95°C for 2 min;
    Stage 2: Set up a thermocycling reaction for 40 cycles using denaturation at 95°C for 30 s and extension at 55°C for 1 min; and
    Set up data collection at the extension step.
13. Save the settings and start the reaction.
14. After the reaction, go to the "File menu" and choose "Export". Then choose "Multicomponent" and SDS 2.2 format.

3.3. Data Analysis

The data from the 9700 HT instrument is analyzed using the provided Excel macro. The QEXT data from other instruments can be analyzed using the same formulae as provided in the macro (*see* **Note 6**). The following procedure should be followed when analyzing the QEXT data with the provided Excel macro.

1. Import the text file containing the multicomponent data (*see* **Note 7**).
2. Click on the tools menu and then on "Calculate average." Two new sheets, called "Mean data" and "Transposed," will appear. Go to the "Transposed" data window and push the button "Run analysis." Then, a new window will appear.

3. Select the column corresponding to the probe used. Then select repeat start 1 and repeat end 25. Select "Delta outcome," and give the maximum difference in fluorescence between unlabeled probes, and probes with a 100% incorporation in the text box (*see* **Note 8**). Select a "Descending curve" and click on "Find efficiency."
4. A new sheet will appear showing the results for each well. The "Effect" shows the relative fraction of unlabeled probes after one labeling cycle. The "Min_value" shows the relative fraction of unlabeled probes after the completed labeling reaction, whereas R2 shows the correlation coefficient (R^2). This value indicates the quality of the prediction. The row labeled "Value/repeat" shows the repeat number, the "Real value" row shows the measured values, and the row labeled "Fitted values" shows the values from the prediction.

3.4. Data Interpretation

1. The incorporation rate per cycle is one minus the "Effect." The incorporation rate should be proportional to the amount of template in the sample. By dividing the incorporation rate in a given sample by the rate determined in a 100% reference sample, then one should obtain the fraction of the target relative to the reference.
2. The "R2" shows the squared regression coefficient (R^2), which indicates the accuracy of the prediction. The R^2 value will be very low if there is no target or no labeling. A high incorporation rate > 0.05, and a low R2 < 0.8 indicates that there is something wrong with that particular sample.
3. The accuracy of the prediction also can be inspected visually by using the standard graph options provided in Microsoft Excel. Deviations between the determined and the predicted values indicate that there is something wrong in the analysis. There is either an error with the actual reaction, or the prediction is wrong (*see* **Note 9**).

4. Notes

1. The first development of the QEXT system was done on an ABI PRISM 7700 (Applied Biosystems). The protocols presented here can be directly adapted to that machine. Unfortunately, however, our Microsoft Excel macro has not been adapted to data from ABI PRISM 7700.
2. The first nucleotide following the 3´-end should be a discriminatory cytosine (SNP allele) unique to the target group. The melting temperature (Tm) of the QEXT probe should be between 55 and 65°C, and the probe preferably between 18 and 30 nucleotides. The probes are quite flexible with respect to Tm because the specificity in the QEXT assay is in the probe labeling, and not in the hybridization.
3. Most likely, the protocol would work equally well the AmpiTaq FS (Applied Biosystems). Unfortunately, however, this enzyme is not sold separately, but as a part of a DNA sequencing kit.
4. It should also be possible to use other quencher-labeled dideoxy nucleotides in the assay. We have already evaluated quencher-labeled dideoxy uracil. The performance was approximately similar to the quencher-labeled dideoxy cytosine.

5. The amount of QEXT probe can be optimized. The probe concentration is a tradeoff between incorporation efficiency and signal-to-noise ratio. A higher probe concentration gives a higher signal-to-noise ratio but a lower incorporation efficiency. The signal for the unincorporated probe should be between 1000 and 2000 RFU.
6. The macro has been for in-house use only and may not work properly under other environments. Particularly, one should be aware of the difference between comma and period in European and American style in denoting decimals. The source code, however, is freely available for further modifications. The users also can modify the Visual Basic macro to comply with other instruments than 9700 HT.
7. It is important to ensure that the data are imported as numbers. If there are problems, the regional setting of the computer should be set to US-style.
8. A sample should be included that contains 100% of the given target. The asymptote for maximum incorporation can be determined from this sample.
9. We have observed two particular artifacts that can occur. If there is evaporation in the sample, then all the signals will increase during the run. The other artifact that we have observed is an increase in the fluorescence signal for the reporter in some of the samples during the first few cycles. This artifact could be attributable to autofluorescence of the exonuclease. The samples containing this pattern should either not be analyzed or the initial fluorescence value should be extrapolated and entered manually in the Excel sheet.

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10

Amplified Fragment-Length Polymorphism and Protein Profiling for Identification of *Campylobacter lari* Subgroups

Birgitta Duim and Jaap Wagenaar

Summary

Amplified fragment-length polymorphism analysis (AFLP) has been shown to be a suitable method for subtyping of bacteria belonging to the genus *Campylobacter*. *Campylobacter lari* is a phenotypically and genotypically diverse species that comprises the classical nalidixic acid-resistant thermophilic campylobacters and the biochemical *C. lari* variants, urease-positive, nalidixic acid-susceptible, and urease producing nalidixic acid-susceptible strains. AFLP profiling and whole-cell protein profile analysis are suitable methods for studying the taxonomic and epidemiological relationships among strains of the *C. lari* variants. Numerical analysis of AFLP profiles and of partial protein profiles allows the discrimination of distinct *C. lari* genogroups. No correlation of these genogroups with different sources of the strains has been identified until now.

Key Words: *Campylobacter lari*; AFLP typing; genogroups; protein profiling.

1. Introduction

Campylobacter species are important pathogens that cause a variety of diseases in humans and animals ***(1,2)***. The most frequent found are the thermophilic species *Campylobacter jejuni*, *C. coli*, *C. upsaliensis*, and *C. lari* ***(3)***. *C. lari* has been associated in humans with diarrhoea ***(3)***, bacteraemia in immunocompromised and immunocompetent patients ***(4,5)***, urinary tract infection ***(6)***, reactive arthritis (***7***) and, recently, with a prosthetic joint infection ***(8)***. Infections after consumption of contaminated shellfish as well as a large outbreak from a waterborne common source have been reported ***(9,10)***. Outbreaks caused by *C. lari* incidentally are reported and it is assumed that the number of *C. lari* infections is

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highly underreported as the isolation methods for Campylobacter species are developed for the detection of *C. jejuni* and *C. coli* ***(11)***. *C. lari* is widely distributed in the environment and can be isolated from a variety of sources including water and animals. Seagulls have been shown to be a reservoir for *C. lari* and have been proposed to contribute to the contamination of water storage reservoirs and of mussels and oyster banks ***(12,13)***.

Since the late 1980s, the identification of *C. lari* has been performed using nalidixic acid with the production of urease. Primarily nalidixic acid-resistant thermophilic *Campylobacter* (NARTC group) and nalidixic acid-susceptible strains (NASC strains) were identified. Subsequently, urease-producing strains (UPTC strains) and urease-producing nalidixic acid-susceptible strains (UP-NASC ***[13–15]***) were identified.

Because the phenotypic diversity and the contribution of *C. lari* phenotypes in human infection is incompletely understood, characterization of the *C. lari* strains that are present in the environment and in human diseases is important. Furthermore, typing may identify strain or group specific markers which may contribute to their pathogenic potential. We describe in this chapter the method we have developed for molecular identification and typing of *C. lari* isolates obtained from various sources (birds, environmental samples, and human patients). Once bacterial strains are isolated they are typed with amplified fragment-length polymorphism analysis (AFLP) and protein profile analysis. The AFLP technique relies on selective amplification of restriction fragments from a digest of genomic deoxyribonucleic acid (DNA). Two restriction enzymes are used to digest DNA, giving rise to fragments of a size (often 50–500 bp) suitable for resolution on polyacrylamide gel or capillary DNA sequencers. Double-stranded adapters, specific for either restriction site are ligated to the DNA fragments, serving as target sites for primers used in PCR amplification. To reduce the number of amplified bands, selective nucleotides can be synthesized at the 3´ ends of the PCR primers, allowing amplification of only a subset of the DNA molecules (*see* **Fig. 1**). Initially, the AFLP utilized radioactively labeled primers for PCR amplification of small genomic fragments ***(16)***. More recently, fluorescent-labeled AFLP patterns are analyzed on gel-based or capillary DNA sequencers, with the corresponding advantage being that the resulting fingerprints may be directly and easily imported into software programs and stored in databases for further processing, for example, comparisons of similarity.

We have adapted the AFLP method for typing of *Campylobacter* strains, and identification of *Campylobacter* species ***(17)***. The AFLP method appeared to correlate with typing date obtained with other genetic typing methods ***(18)*** and was demonstrated to consist of a good discriminatory power for typing of *C. lari* strains ***(19)***.

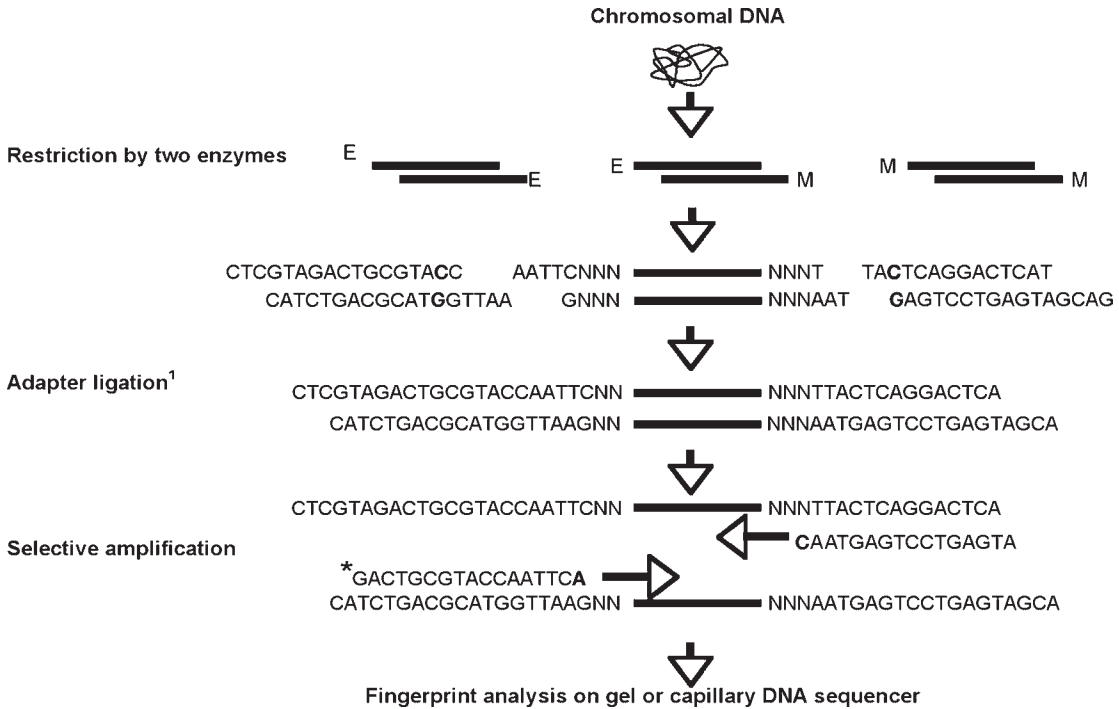

Fig. 1. Schematic representation of the amplified fragment-length polymorphism method. In the adapter sequence ([1]), a point mutation (in bold) is incorporated to prevent digestion after ligation. Both polymerase chain reaction primers contain one selective nucleotide (in bold). One primer is fluorescently labeled (*). (From **ref.** ***19*** with permission from ASM.)

Polyacrylamide gel electrophoresis (PAGE) in the presence of the detergent sodium dodecylsulfate (SDS) has proved to be an important method for the analysis of the protein composition of tissues, cells or biological membranes. From the mid 1970s, electrophoresis of whole-cell lysate fractions in slab gels became an attractive tool for the taxonomic positioning and epidemiological typing of microorganisms and standardized protocols arose to make gels amenable for computerized analysis ***(20,21)***. Currently, SDS-PAGE still holds an important place in the taxonomic characterization of microorganisms to strain level and species ***(22)***. In this chapter, we describe a method for protein profile analysis of *C. lari*. Analysis of a protein range with numerical analysis enables the identification of *C. lari* genogroups ***(19)***.

AFLP has the advantage that it samples a random proportion of the whole genome and therefore has the potential to provide high-resolution epidemiological typing. With AFLP a genotype of an individual *C. lari* is obtained that may cluster into different genotypes. The method described in this chapter is suitable for typing of strains of several *Campylobacter* species. In general, the AFLP method is applicable to almost all microorganisms without previous knowledge of chromosomal DNA or restriction enzyme digestion sites. Moreover, the method is straightforward and can be completed within 8 to 48 h (depending on the electrophoresis platform used). The most important control steps in the method are the purity and quantity of DNA. Data analysis may be performed using software packages as patterns from polyacrylamide gels or capillary sequencers are too complicated to analyse manually. Analysis of protein profiles, with numerical analysis of restricted parts of the profiles results in clustering of *C. lari* strains (*see* **Fig. 2**). When a similar clustering of strains is obtained by phenotypic and genotypic methods, it supports the definition of a specific genogroup. In summary, AFLP and protein analysis are both methods that are general and reproducible, which can be performed by any laboratory.

2. Materials

2.1. Isolation of Campylobacter lari

1. Blood agar plates supplemented with 5% sheep blood.
2. Anaerobe gas packs or an Anoxomat system (Mart B.V., Lichtenvoorde, The Netherlands) to create a microaerobic atmosphere.
3. Heart infusion broth with 15% glycerol.

2.2. Isolation of DNA

AFLP requires only a small amount of purified chromosomal DNA which may be isolated by general methods, such as the Boom et al. method ***(23)***. Commercially DNA-extraction kits, for example, Wizard kit (Promega), Qiagen columns (Qiagen), Puregene kit (Genta Systems), MagnaPure (Roche), also are available.

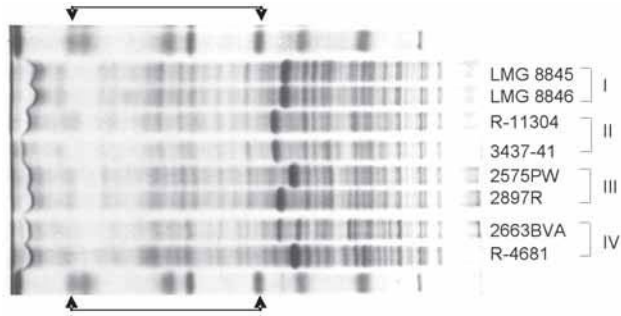

Fig. 2. Whole-cell protein profiles of two representative strains from a *C. lari* genogroup. Roman numbers correspond with clusters defined by numerical analysis of the region indicated by arrows. The molecular mass markers used (top and bottom lanes) were β-galactosidase (116 kDa), bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20,1 kDa; the latter is observed as a double band), and lysozyme (14,2 kDa). (From **ref. *19*** with permission from ASM.)

2.3. Restriction of DNA and Ligation of Adapters

1. Restriction-enzymes *Hind*III 10 U/µL (Promega) and *Hha*I 10 U/µL (New England Biolabs; *see* **Note 1**).
2. Restriction site-specific adapters: *Hind*III adapters: 5´ CTC GTA GAC TGC GTA CC 3´/5´AGC TGG TAC GCA GTC 3´ (4 pmol/µL), *Hha*I adapters: 5´ GAC GAT GAG TCC TGA TCG 3´/5´ ATC AGG ACT CAT CG 3´ (40 pmol/µL).

2.4. PCR

1. PCR primers for preselective PCR are based on the adapter sequences: *Hind*III 5´ GAC TGC GTA CCA GCT T 3´ (5 pmol/µL) and *Hha*I 5 GAT GAG TCC TGA TCG C 3´ (50 pmol/µL; *see* **Note 2**).
2. PCR primers for selective PCR are: *Hind*III 5´ 6-carboxyfluorescein GAC TGC GTA CCA GCT TA 3´, *Hha*I 5´ GAT GAG TCC TGA TCG CA 3´.
3. PCR buffer: 10 m*M* Tris-HCl, pH 8.3, 50 m*M* KCl, 2.5 m*M* $MgCl_2$, and 200 µ*M* of each dNTP.
4. Taq polymerase: AmpliTaq polymerase (Applied Biosystems).

2.5. Acrylamide Gel Electrophoresis for ABI 373A

1. Dilution buffer (1X TE buffer): 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0.
2. Loading buffer: 1.25 µL of deionized formamide, 0.25 µL of blue dextran/50 m*M* EDTA loading solution and 0.5 µL of GeneScan-500 [ROX] size standard (Applied Biosystems; *see* **Note 3**).
3. Analyze fragments on a 7.3% sequencing gel on the ABI 373A DNA sequencer (Applied Biosystems).

2.6. Whole-Cell Lysate Preparation

1. Phosphate-buffered saline, pH 7.3.
2. Solvent mix reagent containing: 0.0625 *M* Tris-HCl, pH 6.8, SDS 2% (w/v), glycerol 10% (v/v), bromophenol blue 0.001% (w/v), β-mercapto-ethanol 5% (v/v).

2.7. Protein Gel Analysis

1. Discontinuous gels are run in a vertical slab gel apparatus. Gels are usually 1.0- to 1.5-mm thick. The separation gel is 12.6 cm long and contains 10 or 12% acrylamide (10 or 12% T) with 2.67% crosslinking (2.67%). The final concentration of other components in the separation gel is 0.375 *M* Tris-HCl, pH 8.8, and 0.1% SDS. The stacking gel is 12-mm long and contains 5% T with 2.67% C, 0.125 *M* Tris-HCl, pH 6.8, and 0.1% SDS.
2. All solutions are made with distilled or Milli-Q water.
3. Acrylamide solution: combine acrylamide 29.2 g and *N,N´*-methylene-bisacrylamide 0.8 g in water. Adjust to 100 mL (*see* **Note 4**).
4. Separation gel buffer: add 18.15 g of Tris to 50 mL of water and adjust to pH 8.8 with HCl. Make up to 100 mL with water.
5. Stacking gel buffer: add 6 g of Tris to 50 mL of water. Adjust to pH 6.8 with HCl and make up to 100 mL with water.
6. 10% SDS solution: add 10 g of SDS to 100 mL ot water.
7. Ammonium persulfate (APS): combine 1 mL of water and 0.1 mg of APS (*see* **Note 5**).
8. *N,N,N´,N´*-tetra-methylethylene diamine (TEMED) and water-saturated isobutanol.
9. Upper and lower reservoir buffer: combine 12 g of Tris, 57.6 g of glycine, and 4 g of SDS in 4 L water, pH 8.3 (*see* **Note 5**).
10. A molecular mass marker with size range of 14–116 kDa.
11. Fixation: 3% trichloroacetic acid.
12. Staining: 0.25% Coomassie blue R-250 in 50% (v/v) methanol and 10% (v/v) acetic acid.
13. For removal of excess stain: 25:10 (v/v) methanol:acetic acid.

2.8. Data Analysis

1. GeneScan Software (Applied Biosystems) is used for detection of AFLP fragments after electrophoresis.
2. The GelCompar v 4.1. or BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium) is used for direct import of data for normalization, background subtraction, and cluster analysis of AFLP profiles and for densitometric analysis, normalization and interpolation, and numerical analysis of the protein profiles (*see* **Note 6**).

3. Methods

3.1. Bacterial Cultures

1. Grow bacteria on blood agar plates at 37°C for 2–3 d. Incubate in a microaerobic atmosphere consisting of approx 7% CO_2, 6% O_2, 7% H_2, and 80% N_2.

2. Bacterial cultures should be stored at –80°C in 15% (w/v) glycerol in heart infusion broth.

3.2. AFLP Analysis

3.2.1. Isolation of DNA

1. Collect colonies from the plate with a swab and resuspend in 1 mL of sterile water. DNA is isolated according to the procedures outlined in the Puregene kit or other commercially available kits.
2. Estimate the DNA concentration by agarose gel electrophoresis and store DNA at a concentration of 100 ng to 1 µg/µL in sterile water.

3.2.2. AFLP Reaction

1. Perform restriction/ligation in a mix containing 2 µL of 20 to 50 ng purified genomic DNA, 2 µL of *Hind*III adapters, 2 µL of *Hha*I adapters, 0.5 µL of 1.0 mg/mL bovine serum albumin, 1.0 µL of 0.5 *M* NaCl, 1.5 µL of 10X T4 DNA ligase buffer containing [ATP], 0.5 µL of 10 U/µL *Hind*III, 0.5 µL of 10 U/µL *Hha*I, and 1.0 µL of 1 U/µL T4 DNA ligase. Incubate at 37°C for 2 h.
2. Dilute the reactions by adding 186.0 µL of H_2O.
3. Of the diluted mix, use 4 µL in a preselective amplification mix containing: 1.0 µL of *Hind*III preselective primer, 1.0 µL of *Hha*I preselective primer, 2.0 µL of 10X PCR buffer, 2.0 µL 25 m*M* $MgCl_2$, 1.0 µL of dNTPs, 0.25 µL of Taq DNA polymerase, and 8.75 µL of H_2O.
4. Amplify using the following conditions (preselective PCR): 1 cycle of 2 min at 72°C and 20 cycles of 20 s at 94°C, 30 s at 56°C, and 2 min at 72°C.
5. Amplify 1.5 µL of preselective amplification product with the mix containing: 1.0 µL of *Hind*III selective primer, 1.0 µL of *Hha*I selective primer, 1.0 µL of 10X PCR buffer, 1.0 µL of 25 m*M* $MgCl_2$, 1.0 µL of dNTP's, 0.25 µL of Taq DNA polymerase, and 3.25 µL of H_2O.
6. Selective PCR amplification conditions as follows should be used: 10 touch down cycles of 20 s at 94°C, 30 s (with the annealing temperature being reduced by 1°C per cycle from 66°C to 57°C), and 2 min at 72°C.
7. Follow immediately with 20 cycles of 20 s at 94°C, 30 s at 56°C, and 2 min at 72°C with a final incubation of 30 min at 60°C.
8. Dilute PCR product 1:1 (vol/vol) in the buffer, mix 1.0 µL of the diluted selective amplification product with 1.25 µL of deionized formamide, 0.25 µL of blue dextran/50 m*M* EDTA loading solution, and 0.5 µL of GeneScan-500 [ROX] size standard. Load 3 µL on a 7.3% denaturing sequencing gel on an ABI373A sequencer.
9. Run at 2500 V for 5 h.

3.2.3. Computer Analysis of AFLP Patterns

1. During electrophoresis, data are collected with the GeneScan software (Applied Biosystems).

2. After tracking and extraction of lanes, import the densitometric curves (fluorescent signal is expressed as a curve) into the GelCompar or BioNumerics software (Applied Maths) to calculate the curve-height (intensity), curve-weight, and curve-area (position). Gels are normalized by use of the reference positions of the internal DNA size marker GS-500.
3. Fragments ranging in size from 50 to 500 bp are used for comparison. The AFLP curves are calculated with the Pearson product-moment correlation coefficient of similarity. Clustering analysis is subsequently conducted by the unweighted pair-group method using arithmetic averages (UPGMA) clustering method (position tolerance 1%, optimization 2%; *see* **Fig. 3**).

3.3. Protein Profile Analysis

3.3.1. Preparation of Whole-Cell Lysates

1. Colonies grown on the blood agar plate are harvested by suspending the colonies in several milliliters of PBS (100 mg cells [wet weight] in approx 20 mL of PBS).
2. Add PBS to achieve a final volume of 30 mL.
3. Centrifuge 20 min at 10,000*g*, discard the supernatant, and wash cells by resuspending them in 30 mL of PBS. Harvest by centrifuging for 10 min at 10,000*g*.
4. Repeat washing and centrifuging at least one more time.
5. After the final centrifugation step, discard the supernatant and dissolve 50 mg of the pellet (wet weight) in 0.9 mL of solvent. Sample preparation can be performed in 1.5-mL centrifuge tubes. Thoroughly mix the suspension.
6. Boil the pellet/solvent solution for 10 min. Finally, centrifuge for 10 min at 7000*g* at 4°C to sediment large fragments and unlysed cells.
7. If not used immediately, store the samples at –20°C for short periods or at –80°C for prolonged periods. If stored, boil pellets before electrophoresis.

3.3.2. SDS-PAGE of Whole-Cell Proteins

1. Assemble clean glass plates with spacers in a cassette (*see* **Note 7**).
2. Add 20 mL of separation buffer, 32 mL of acrylamide solution, 0.8 mL of 10% SDS, and 26.8 mL of water. After mixing add 40 µL of TEMED and 0.28 mL of 10% APS. Mix and pour the solution immediately between the plates. Overlay with 2 mL of isobutanol.
3. After 1 h, discard the isobutanol and fill with separation gel buffer. Cover with parafilm and allow the gel to polymerize for 16 to 24 h.
4. Pour off the liquid layer and rinse the gel surface with water.
5. Mix 5 mL of stacking buffer, 3.4 mL of acrylamide solution, 0.2 mL of SDS, and 11.3 mL of water. Add 25 µL of TEMED and 0.1 mL of 10% APS. Stir thoroughly and pour the solution between the plates. Insert a Teflon comb and remove airbubbles. Allow the gel to polymerize for 30 min.
6. Mark the position of slots and apply the protein samples in each slot with a microsyringe. Apply the size standard in the wells on both sides of the samples.
7. Run the gel overnight at constant current (6 mA per gel) and temperature.

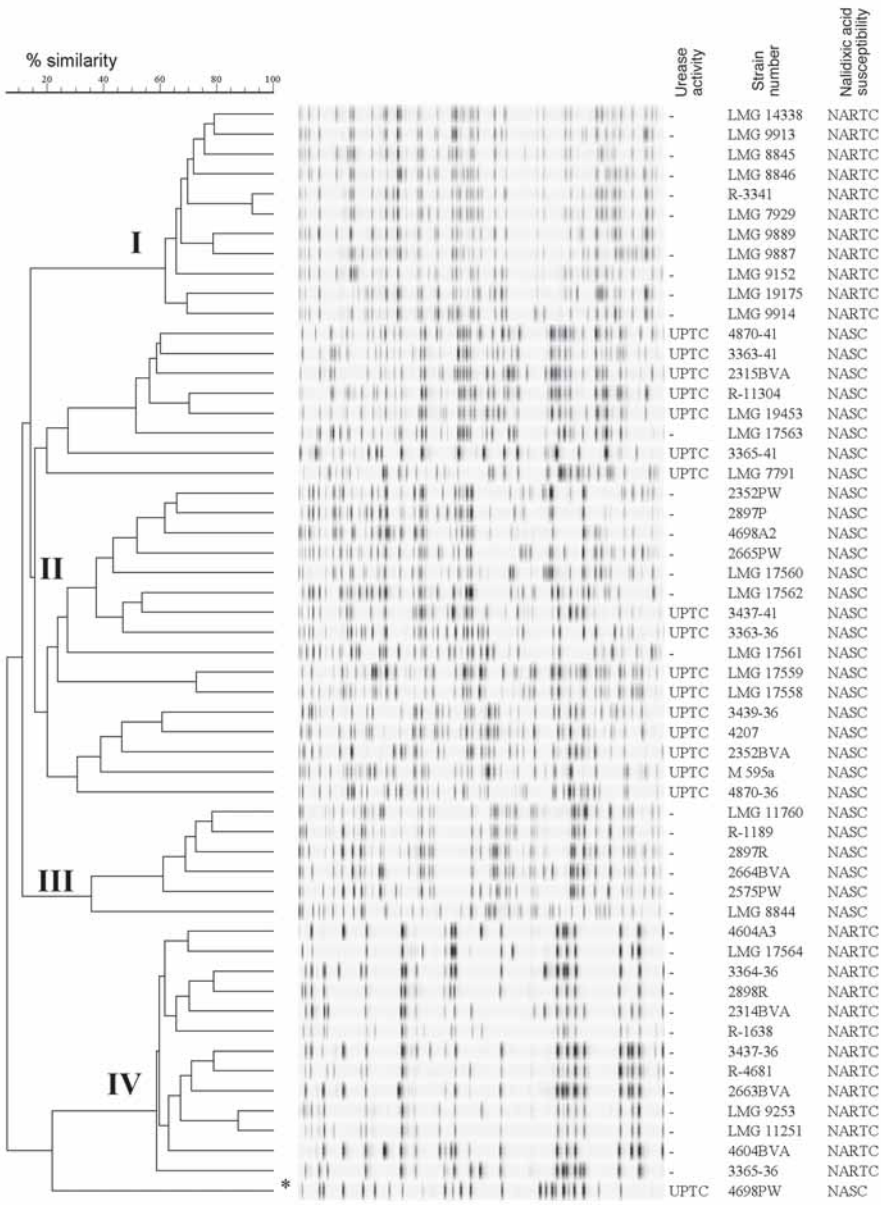

Fig. 3. Dendrogram derived from unweighted pair group method using average linkage (UPGMA) cluster analysis of amplified fragment-length polymorphism profiles (50–500 bp) of *C. lari*. The scale bar indicates levels of linkage between patterns. The roman numerals indicate the distinct clusters to which the strains belong. The asterisk indicates a highly diverse AFLP pattern that clusters separately. (From **ref. *19*** with permission from ASM.)

3.3.3 Gel Fixation and Staining

1. Add the gel immediately for fixation in 3% trichloroacetic acid and shake gently for 30 min.
2. Pour of the solution and add staining solution. Shake gently for 1 h.
3. Destain the gel in a solution containing 25% (v/v) methanol and 10% (v/v) acetic acid.
4. Store the gel in a closed container or dry using a gel dryer system.

3.3.4. Computer Analysis of Protein Profiles

1. Take a picture (high resolution) of the gel using a charge-coupled device camera from the laboratory.
2. Digitize the photographs using a flat bed scanner and store as TIFF files.
3. Import the TIFF files in the GelCompar or BioNumerics software. The gels are normalized using the size standard lanes on both sides of the gel.
4. Only the band sizes between 36 kDA and 20.1 kDa are included in the pearson coefficient for similarity calculation and UPGMA cluster analysis (*see* **Note 8**).

4. Notes

1. Several enzymes have been used, including *Eco*RI, *Pst*I, *Hind*III, and *Apa*I, combined with either *Mse*I or *Taq*I. For the majority of bacterial genomes, a combination of *Eco*RI and *Mse*I appears to be the most suitable for AFLP analysis, although not for *C. jejuni* (because of the fact that *Eco*RI digestion is inhibited in this species ***[24]***).
2. Compared with a single selective PCR, a preselective AFLP before a selective PCR results in less variation in peak intensities. Also, the stringent PCR annealing temperature yields high reproducibility in the AFLP analysis.
3. Other gel or capillary DNA sequencers and several fluorescently labeled dyes and size markers are available.
4. Acrylamide is neurotoxic and should be handled with care. Store dark at 4ºC. The separation gel buffer and stacking gel buffer are stable for at least 2 wk when stored at 4ºC.
5. APS and the upper reservoir buffer must be freshly prepared before use. The lower reservoir buffer can be used for several weeks.
6. AFLP data may be directly imported into GelCompar or BioNumerics software packages (Applied Maths) when ABI (Applied Biosystems), ALF, or MegaBace (Amersham Biotech) platforms are used. Other platforms require import from digitized images in a standard graphical file (e.g., TIFF).
7. SDS-PAGE analysis of whole-cell lysates is performed according to Pot et al. ***(21)***.
8. The high molecular protein bands are highly homogenous and only when a restricted protein region (20.1–36 kDa) is analyzed differentiation in genogroups is obtained.

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11

Use of Peptide Nucleic Acid Probes for Rapid Detection and Enumeration of Viable Bacteria in Recreational Waters and Beach Sand

Nwadiuto Esiobu

Summary

Environmental monitoring and public health risk assessments require methods that are rapid and quantitative with defined sensitivity and specificity thresholds. Although several molecular techniques have been developed to rapidly detect bacteria in complex matrices, the challenge to simultaneously detect and enumerate only viable cells remains a limiting factor to their routine application. This chapter describes the use of peroxidase-labeled peptide nucleic acid (PNA) probes to simultaneously detect and count live *Staphylococcus aureus*, a human pathogen in sea water and beach sand. Mixed bacteria from the environmental sample were immobilized on polyvinylidene difluoride membrane filters and allowed to form microcolonies during a 5-h incubation on Tryptic soy agar plates. PNA probes targeting species-specific regions of the 16S rRNA sequences of *S. aureus* were then used to hybridize the target bacteria *in situ*. Probes were detected by capturing chemiluminiscence on instant (e.g., Polaroid) films. Each viable cell (i.e., rRNA producing) is detected as a light spot from its microcolony on the film after scanning the image into a computer. This rapid *in situ* hybridization technique is simple and highly sensitive and could be developed into portable kits for monitoring pathogens and indicators in the environment.

Key Words: *Staphylococcus aureus*; environmental monitoring; 16S rRNA sequences; PNA probes; *in situ* hybridization; beach sand; sea water; quantitation; plate counts.

1. Introduction

Conventional methods for monitoring pathogens and indicator bacteria in the environment often are based on biochemical fingerprinting using multiple tube fermentation, membrane filtration, and chromogenic substrates ***(1)***. These techniques are grossly limited by lack of specificity, lengthy incubation time, and

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poor detection of slow growers and noncultivable forms ***(2)***. Extended turnaround time of the standard practice ***(3)*** renders pollution source-tracking difficult, especially during sporadic events, and could lead to erroneous risk assessment. Recent advances in polymerase chain reaction (PCR) techniques such as nested PCR ***(4,5)*** and PCR–enzyme-linked immunosorbent assay have considerably improved sensitivity and detection limits of bacteria in water. Indeed, real-time detection and quantitation methods based on multiplex PCR and reverse-transcription PCR techniques have been developed ***(6,7)***. Other methods include the quantitative detection of microbial genes by deoxyribonucleic acid (DNA) microarrays ***(8)***. However, serious limitations, such as the high technicality of procedures, uncertain viability status of detected bacteria, and multiple environmental interferences with signal detection, hamper the widespread use of these elegant techniques.

We have adapted the novel peptide nucleic acid (PNA)–chemiluminiscent *in situ* hybridization (PNA–CISH) to simultaneously detect and count only viable target bacteria within 7 h ***(9)***. Unique probe signatures are used to target species-specific 16S ribosomal ribonucleic acid (rRNA) sequences of bacteria. Ribosomal RNA is highly conserved among closely related species, and differences between these highly conserved rRNA sequences enable a definitive identification of target microorganisms ***(10)***. High cellular abundance, universal distribution, and use as a phylogenetic marker are some of the reasons why rRNA is a preferred target for *in situ* probe-based assays. PNA molecules are true mimickers of DNA in terms of base-pair recognition. A neutral polyamide molecule replaces the pentose phosphate backbone, eliminating the charge repulsion between PNA strand and the target RNA ***(11)***. This pseudo peptide allows improved hybridization characteristics, such as increased target specificity, higher thermal stability, binding independent of salt concentration, and improved binding kinetics. The hybrid chemical structure of PNA–DNA and PNA–RNA also are not recognized by nucleases or proteases; thus, the signal has an extended lifetime in vivo and in vitro ***(12)***. PNAs hybridize to complementary DNA or RNA in a sequence-dependent manner, according to the Watson–Crick hydrogen bonding scheme**.** Unlike DNA, PNA probes can bind in either parallel or antiparallel fashion and to single- or double-stranded DNA or RNA ***(11)***, rendering them particularly flexible and versatile in probe technology. These probes could be used in multiple detection protocols and platforms, such as CISH or fluorescent *in situ* hybridization (FISH).

Staphylococcus aureus is a well-documented human opportunistic pathogen that is responsible for a number of skin infections ***(13)***. The prevalence of *S. aureus* in the environment could be an indicator of bather density and risk of crossinfection among individuals on beach sand and bathers in the ocean and public pools ***(14,15)***. Selective-differential media provide a presumptive detection of staphylococci with a high degree of error and false-negative results. Not-

withstanding, culture plate counts may be used as a reference for the more sensitive and specific PNA probe technique. The 16S rRNA sequence specific for *S. aureus* has been published ***(9,16)***, and its use allows a rapid documentation of the prevalence of the organism after extraction in a buffer (for sand samples), membrane filtration, microcolony production on complex agar, and *in situ* hybridization and detection protocols. This method is particularly advantageous because the detected cells could be allowed to develop into cultures used for physiological, resistance, and virulence studies where needed.

2. Materials

2.1. Sample Collection

1. Sterile screw capped vials (at least 500-mL capacity).
2. Disinfected hand trowels.
3. Sterile resealable bags (e.g., Ziplock®) for sand samples.
4. A refrigerated cooler for transport of samples.

2.2. Sample Preparation

2.2.1. Extraction of Bacteria

1. 50- to 250-mL Capacity sterile vials.
2. 10X Phosphate buffered saline (PBS): 1.37 *M* NaCl, 27 m*M* KCl, 100 m*M* Na_2HPO_4, and 18 m*M* KH_2PO_4. Adjust to pH 7.4 with HCl if necessary. Autoclave before storing at room temperature. If crystals form, warm solution before preparing a 1X by mixing 1 part to 9 parts of sterile water.
3. Multispeed vortex machine.

2.2.2. Membrane Filtration and Growth of Microcolonies

1. 25- to 1000-mL Measuring cylinder.
2. Tryptic soy agar (TSA) plates.
3. Sterile 50-mm diameter polyvinylidene difluoride membrane filter with grids (0.22-µm pore size).
4. Membrane filtration manifold/flask and vacuum pump (Fisher).
5. Microfil V funnels (Fisher).
6. Sterile pair of forceps.
7. Distilled or Milli-Q^{Æ} water.
8. Incubator at 37°C.
9. S110 Agar.

2.3. In Situ *Hybridization With PNA Probes*

1. 16S rRNA sequence specific probe for *S. aureus* (5´–3´) GCT TCT CGT CCG TTC (Applied Biosystems; *see* **Note 1**).
2. Fixation solution: 0.35% (v/v) gluteraldehyde, 0.01% (w/v) urea-H_2O_2, 5 m*M* NaN_3, 90% denatured ethanol. Prepare a 1X solution and store at 2 to 8°C. Do not freeze.

3. Cellulose pads.
4. Empty disposable Petri dishes. Use small 50-mm plates to save reagents and improve contact with probe.
5. Petri slides (Boston Probes Inc.).
6. Hybridization buffer: 25 m*M* Tris-HCl, pH 9.5, 50% (v/v) formamide, 0.7% (v/v) Tween-20, 2% (w/v) polyvinylpyrrolidone, 1% (w/v) yeast extract, 1% (w/v) casein, 0.1 *M* NaCl, 5 m*M* ethylene diamine tetraacetic acid. Store in the refrigerator (*see* **Note 2**).
7. Wash buffer: 10 m*M* CAPSO, pH 10.0, 0.2% Tween-20. Solution may need to be heated on a stirring hot plate to dissolve. Store at room temperature. The solution must be warmed up to 50°C during probe wash. Adjust pH with NaOH as needed.
8. Wash racks and troughs (*see* **Note 3**).
9. Incubator at 50 to 55°C.
10. Water bath at 50°C.
11. Timer.

2.4. Detection of Probes

1. Chemiluminescent substrates: luminol/enhancer and stable peroxide (Pierce Chemical).
2. Fotolopes.
3. Spotlight™ Camera (Polaroid).
4. Polaroid instant films ASA 20,000.
5. A computer equipped with CanoScan software or any other scanning device.

2.5. Quality Assurance

1. Positive control: *S. aureus* ATCC 6538 ***(16)***. Use a log phase culture of a known density (*see* **Note 4**).
2. Negative control: autoclaved environmental samples at 15 psi, 120°C for 20 min. Allow to cool to room temperature before use.
3. Background interference controls: nonautoclaved samples without probes.
4. TSA plates for estimation of colony-forming units.
5. Microscope slides and cover slips, immersion oil for fluorescence, polycarbonate membrane, and DAPI (Molecular Probes). *See* **Fig. 1** for the portable equipment and materials required.

3. Methods

Sample quality and sampling strategies are as important as detection techniques in environmental monitoring. In beach sand, for example, the distribution of *S. aureus* varies widely within one square foot area (unpublished data). Bacterial numbers in recreational waters also fluctuate with tides and human usage in 1 d. In addition, because healthy people can be carriers for this bacterium, it is important that sampling is aseptically performed and analyzed with a minimum of delay. Samples must be representative of the niche so that reproducible data are generated.

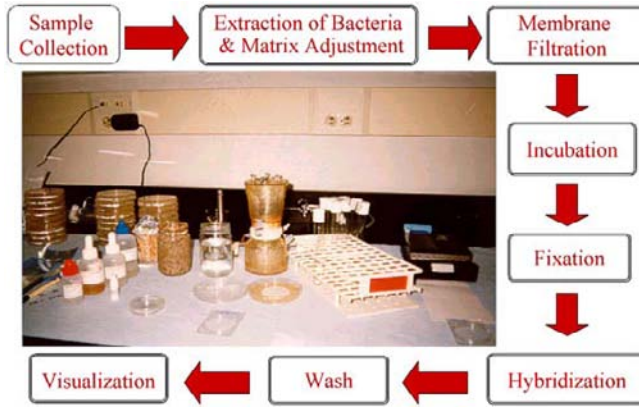

Fig. 1. Some simple, portable, and easy-to-use materials for peptide nucleic acid–chemiluminiscent *in situ* hybridization techniques and steps involved in diagnostic bacteriology.

3.1. Collection of Samples

1. Collect at least five sand samples from a square meter transect by means of hand trowels (disinfected with 10% bleach and rinsed in sterile water) from the top 10 cm depth.
2. Mix all five samples thoroughly in a sterile resealable (e.g., Ziplock) bag or vial to create a composite sample of that replication
3. Collect recreational water samples in dark 500-mL bottles at least 10 cm below the water surface.
4. Transport samples to the laboratory on ice and process within 3 h for the most representative and reproducible results.

3.2. Preparation of Samples

3.2.1. Extraction of Bacteria

1. Weigh a well-mixed sand sample into sterile 1X PBS to achieve a 1/10 dilution (e.g., 5 g into 45 mL of PBS).
2. Close the vial and vortex vigorously at moderate to high speed for 60 s (*see* **Note 5**).
3. Clarify the soil extract by allowing it to settle for not more than 1 min.
4. Bacteria will sediment if sample stands for a prolonged period of time.
5. Do a serial 10-fold dilution of sample in 99 mL of PBS diluents to further eliminate interference from sand debris. Immediately plate aliquot samples on TSA or any other complex medium to calibrate the dilutions (*see* **Note 4**).
6. Proceed immediately to membrane filtration using the appropriate dilution.
7. For sea water samples, vortex for approx 60 s to dislodge and disperse bacteria from suspended aggregates.

8. Use a predetermined volume for the membrane filtration step. Before the actual experiment, obtain samples from niche of interest, vacuum filter different volumes of the sample, e.g., 10, 50, 100, and 200 mL on a membrane, and allow them to grow overnight on agar. Select the volume that yields well isolated colonies for membrane filtration step.

3.2.2. Membrane Filtration and Growth of Microcolonies

1. Set up the filtration manifold and funnels as directed by manufacturer. Then, connect to vacuum. Aseptically position the sterile grided membrane filter at the base if using the traditional filtration flask. (Microfil V funnels come with membranes.)
2. Measure appropriate volume of sample into the funnel.
3. Apply vacuum to filter sample.
4. Disengage vacuum and aseptically remove the bacteria-laden membrane with a dry pair of forceps.
5. Gently place the membrane filter (with the bacteria side up) on a TSA plate.
6. Incubate plates at 35 to 37°C for 4 to 5 h in an inverted position to prevent drops of condensates from forming on the membrane (*see* **Note 6**).

3.3. In Situ *Hybridization With PNA Probes*

3.3.1. Fixation of the Microorganisms

1. Place the absorbent cellulose pads in sterile Petri dishes.
2. Soak each one with 1.5 mL of fixation buffer. Be careful not to flood the plate or allow the pads to drip.
3. Using a pair of forceps, carefully transfer the membrane filters from **Subheading 3.2.2.** to the pads with the bacteria side up. At this point the microcolonies, barely visible to the naked eyes, have been formed by a mixed flora on the membrane.
4. Cover the plates and incubate at room temperature for 5 min.

3.3.2. Hybridization

1. Prepare PNA probes by reconstituting the labeled oligonucleotide in aqueous solution according to manufacturer's instruction (Applied Biosystems Inc) (*see* **Note 1**)
2. Mix an appropriate volume of probes with 1.5 mL of hybridization buffer to obtain a probe concentration of 5 n*M*. For example, if the original probe stock was prepared at a 2.5 µ*M* concentration, add 3 µL of the stock to 1.5 mL of hybridization buffer.
3. Carefully transfer the membrane with the fixed microbes from the fixation plates to the Petri slides containing the probe–bacteria side up.
4. Rock the Petri slide gently until the membrane is submerged in the solution.
5. Cover the membrane and incubate at 50°C for 30 min in a hybridization chamber. If a regular incubator is used, place a cup of water to humidify chamber.
6. At this point, only *S. aureus* species have been hybridized.

3.3.3. Washing to Remove Excess Unbound Probe

1. Place the wash rack or partitioned sieve inside a wash container and fill it with preheated wash solution (approx three-quarters full).
2. Then, place wash set up in a prewarmed water bath.
3. Remove the Petri slides individually from the hybridization chamber, discard the lid, and drain the excess hybridization buffer.
4. Submerge the Petri slides with the membrane in the wash rack containing the wash solution without delay. The membrane must be completely submerged. Add more wash solution if needed. Repeat **steps 3** and **4** for all hybridized membrane.
5. Gently remove the wash container from the water bath and drain the solution from the distal end.
6. Refill the container with pre-warmed wash solution and allow to stand for 7 min in the 50°C water bath. Drain the samples and repeat for a total of four 7-min wash cycles.
7. After the last wash, place the wash container on the bench without draining the wash solution.
8. Process each membrane filter one at a time as described below while keeping the others submerged in the wash buffer.

3.4. Detection of Labeled Probes

Fotolopes are transparent strips with a width of approx 13 mm wider than the diameter of the membranes. They are protected with blue and translucent sheets and adhesives on the underside.

1. Peel off the protective sheet carefully without touching the fotolope.
2. Lay down the fotolope gently and mix 100 µL of Luminol enhancer and 100 µL of stable peroxide (Pierce Chemical) on the clear side.
3. Using forceps, remove the membrane filter from the wash rack and place it face (bacteria side) down.
4. Tap it gently to ensure even distribution of the substrate.
5. Leave at room temperature for 2 min for the chemiluminescent reaction to occur.
6. Close the fotolope and remove excess substrate by pressing gently on the edges.
7. Transfer it to the portable camera box loaded with Polaroid film.
8. Capture the emergent light on a Polaroid film following the manufacturer's instructions (*see* **Fig. 2**).
9. Scan the films with light dots into a computer to facilitate enumeration
10. The number of viable Cells (PNA probe counts) is calculated as follows:

$$\text{Total Number of light spots} \times \text{Dilution factor} \div \text{Volume of diluted sample in filtered buffer (mL)}$$

For example, if 500 µL of the ten-thousandth dilution was added to 200 mL of PBS to achieve even spread of cells on membrane during filtration and if 80 chemiluminescent light spots were detected on the membrane, then the number of viable cells in the sample = $(80 \times 10^4 \div 0.5)$ colony-forming units (CFU)/mL.

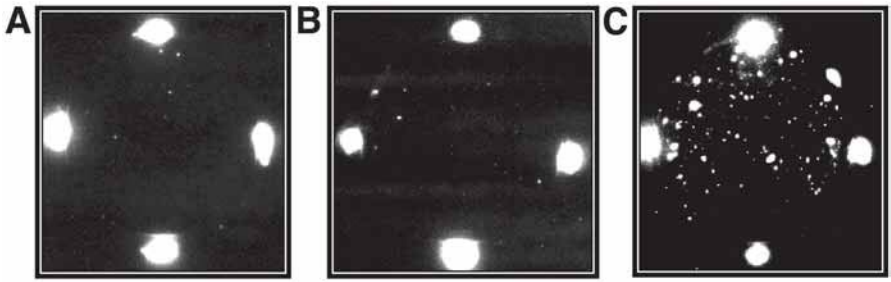

Fig. 2. *Staphylococcus aureus* detected by peptide nucleic acid probes in sea water **(A)**, wet sand **(B)**, and dry sand **(C)**.

3.5. Quality Assurance

Quality assurance tests are conducted to define the sensitivity of probes in the matrix under consideration and their specificity, as well as to delineate possible matrix limitation and interferences with probe detection.

1. Prepare a twofold dilution of a log culture of *S. aureus* in PBS to produce a low-to-medium range population density. Measure the optical density of culture as a starting guide (A_{620} nm values between 0.08 and 0.1 of a log phase culture is approx 100 million cells per milliliter).
2. Introduce the bacteria into sterile sand and sea water to yield numbers like 2, 4, 8, 16, 32 CFUs/gram of sand or 100 mL of water, respectively (CFU is determined by plating on the selective differential agar S110).
3. Prepare samples as described above and correlate PNA detection with CFU determined by plate count.
4. Repeat **step 2** with mixed bacterial strains to test probe specificity.
5. Negative controls and matrix interference tests follow the same protocols **(*9*)**.

4. Notes

1. Custom or published PNA probe sequences can be purchased from www.Applied Biosystems.com. The new PNA probe design software www.appliedbiosystems.com/support/pnadesigner.cfm is a good guide to test new sequences before manufacture. Detailed protocols for in-house manufacture of the probes are cumbersome and have been described previously ***(16)***. Alternative labels to those described in this chapter are also available. Examples include Cye3, modified Fluo tags, and many more. The type of labeling can be selected at purchase. It also is possible, for example, to purchase biotin-coupled probes and then use coupling kits to link an enzyme label.
2. Contains formamide, which is harmful. Appropriate protective clothing must be worn.
3. These could be custom made: 8 cm deep, 8 × 20 cm rectangular sieves with partitions placed in a compatible trough.

4. The dilutions are plated by means of classical microbiological assay. Calibration is by plate count technique correlated to optical density where pure cultures are concerned. This step does not have to be performed every time samples are analyzed. Previous determinations constitute a reasonable guide to decide on which dilutions and volumes to use for the PNA–CISH. The only exceptions are after a storm event and other sporadic episodes.
5. On the one hand, vortexing for longer than 1 min yields lower culture counts and PNA probe counts because of lysis of bacteria. On the other hand, bacteria remain attached to sand crevices when a sample is vortexed for a shorter time, (N. Esiobu unpublished data).
6. If condensates form on the membrane, discard it because it will interfere with subsequent hybridization and detection. Replication is always recommended.

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12

HOOF Prints

Brucella *Strain Typing by PCR Amplification of Multilocus Tandem-Repeat Polymorphisms*

Betsy J. Bricker and Darla R. Ewalt

Summary

A critical component of limiting bacterial disease outbreaks is the tracing of the infection to the index source, which can be facilitated by using a highly discriminating bacterial identification system that will reliably identify genetically related bacterial populations. For pathogenic bacteria with highly conserved genomes, such as the zoonotic pathogen *Brucella*, finding distinguishing markers or traits for strain identification is challenging. This chapter describes a relatively new procedure for identifying *Brucella* strains. The procedure, which is called "HOOF prints" (hypervariable octameic oligonucleotide fingerprints), is based on high levels of polymorphism observed at several genomic loci in the *Brucella* genomes that contain small tandemly repeated deoxyribonucleic sequences. The technique described is designed for medium- to high-throughput analyses. However, the method described can be modified to characterize fewer samples.

Key Words: PCR; *Brucella*; HOOF prints; genotyping; VNTR; capillary electrophoresis.

1. Introduction

Infectious diseases of livestock have a negative impact on the agricultural economy of all countries. A critical component of limiting disease outbreaks is the ability to trace a new outbreak to the original source so that further spread from the index population is prevented. This tracing back requires some type of bacterial identification system that will reliably discriminate between related and unrelated bacterial populations, which is difficult to accomplish for pathogenic bacteria with highly conserved genomes because distinguishing markers or traits are hard to find.

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Brucella species are one example of disease-causing bacteria with highly conserved genomes. Complete genome sequencing of three *Brucella* species has revealed remarkable genetic homogeneity ***(1–3)*** despite differences in host preference. Although some phenotypic characteristics and genetic features have been identified to distinguish among the eight *Brucella* species ***(4–7)***, highly discriminating methods are limited ***(4,8–11)***. Furthermore, some methods have problems with reproducibility from laboratory to laboratory ***(12,13)***.

Recently, small tandemly repeated DNA sequences have become popular sites for finding genetic polymorphisms in bacterial strains because these short tandemly repeated DNAs mutate at a much higher level than the spontaneous mutation rate, mainly as the result of slip-strand mispairing and recombination ***(14)***. This phenomenon has been known for a long time in eukaryotic organisms and is the basis for human DNA identification, but it has only recently been applied to typing pathogenic bacterial strains, such as *Bacillus anthracis* ***(15)***, *Yersinia pestis* ***(16)***, *Haemophilus influenzae* ***(17)***, and *Francisella tularensis* ***(18)***, to name a few.

This chapter describes in detail the procedure for identifying *Brucella* strains based on the high level of polymorphism found at 10 independent genomic loci that contain small tandemly repeated DNA sequences. Because all of the loci contain 8-bp tandem repeats, we have named this technique "HOOF prints," for hypervariable octameric oligonucleotide fingerprints ***(19)***. The procedure presented here is designed for medium- to high-throughput analyses (at least 10 strains or bacterial colonies) and takes advantage of available technologies for rapid and sensitive genetic analyses, including polymerase chain reaction (PCR) and capillary electrophoresis with fluorescent detection. The methods described can be scaled down to characterize a smaller number of samples and can be performed in a modestly equipped laboratory.

2. Materials

2.1. Laboratory Requirements

1. BL-3 facilities approved for work with *Brucella* (if live *Brucella* will be cultured or used).
2. Dedicated work area completely free of contaminating *Brucella* or *Brucella* DNA.
3. Adjustable pipets dedicated to PCR setup only (e.g., P-2, P-10, P-200, and P-1000).
4. Additional pipets (e.g., P-10 and P-200) for dispensing templates and detection procedures (*see* **Note 1**).
5. Disposable pipet tips with aerosol-preventing filters.
6. Multichannel pipets (*see* **Note 2**).
7. Repeating pipettors (e.g., Eppendorf Repeater; *see* **Note 2**).

2.2. Sample Preparation

1. Nutrient agar plates such as tryptose agar (Difco), trypticase soy agar (BBL), or *Brucella* agar (BBL) containing 5% serum (bovine, calf, or fetal calf).

2. Saline: 0.85% (w/v) NaCl in sterile water.
3. CO_2 (10%) incubator or jar.
4. Inoculating loops.
5. Spectrophotometer (e.g., Beckman).
6. Disinfectant (e.g., 1% Lysol IC™, Reckitt & Colman Inc.).

The following items are needed for methanol preservation of cells (optional; *see* **Note 3**).

7. Methanol (reagent grade).
8. Trypticase soy broth with 5% bovine serum.
9. Shaking (rotating) water bath set at 37°C.
10. 50-mL Screw-cap centrifuge tubes.

2.3. PCR Amplification

1. Thermal cycler with the capacity to use 96-well plates.
2. Tabletop centrifuge with plate rotor (optional).
3. 96-Well PCR plates (*see* **Note 4**).
4. Repeating pipettor (*see* **Notes 1** and **2**).
5. Reagents needed for preparing the PCR Master Mixes (*see* **Tables 1–3** for details).
6. PCR-grade water.
7. TE: 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0 (*see* **Note 5**).
8. 10X Reaction buffer: 500 m*M* Tris-HCl, pH 8.3, 100 m*M* KCl, and 50 m*M* $(NH_4)_2SO_4$ at 25°C.
9. 25 m*M* $MgCl_2$.
10. 2.5 m*M* Each of dATP, dCTP, dGTP, and dTTP (Invitrogen).
11. DNA polymerase (*see* **Note 6**).
12. Oligonucleotide primers (*see* **Notes 7–9,** and **Table 1**).
13. PCR cap strips (*see* **Note 10**) or cap mats (*see* **Note 11**).
14. Adhesive plate sealers: foil (AlumaSeal II, Sigma-Aldrich Corp).
15. Plate roller for sealing adhesive plate sealers, cap strips, and cap mats (optional).

2.4. Processing of Amplicons

1. Agarose electrophoresis system (*see* **Note 12**).
2. TBE: 89.0 m*M* Tris-borate, 2 m*M* EDTA, pH 8.3, or TAE: 40 m*M* Tris-acetate, 1 m*M* Na_2EDTA, pH 8.3, electrophoresis running buffer (not required for buffer-less gel systems).
3. Agarose gels, 4% (*see* **Note 12**).
4. Multichannel pipettors and compatible filter tips (*see* **Notes 1** and **2**).
5. Ethidium bromide staining solution: 500 µg/L in running buffer or SYBR Gold Nucleic Acid Gel Stain (Invitrogen; *see* **Notes 13** and **14**).
6. Loading buffer with tracking dyes (Amresco Inc.).
7. Molecular weight markers: 25-bp DNA ladder and 10-bp DNA ladder (Invitrogen).
8. Camera with Wratten no. 22A filter or digital reader for recording gel images.
9. 96-Well PCR plates for diluting amplicons.

Table 1
Primer Sequences

Primer name	5′ to 3′ sequence	1000 X [a]
VNTR-1	**HEX**·GGT GAT TGC CGC GTG GTT CCG TTG AAT GAG	2.01 µg/µL
VNTR-2	**HEX**·CCC GCA TGA TCC GCG AAC AGC TGG ATG	1.80 µg/µL
VNTR-3	**NED**·CAG GCG CTT GAG GAT GAG GCG GCA G	NA[b]
VNTR-4	**6-FAM**·GCA GAA TTT TCG AGG CAT TCG GCG ATG	1.78 µg/µL
VNTR-5	**6-FAM**·GTG CTC CAG GGC GCC GGG AGG TAT GTT TAG	1.98 µg/µL
VNTR-6	**NED**·GCC GCA GGA AAG CAG GCG ATC TGG AGA TTA TC	NA[b]
VNTR-7	**6-FAM**·CAG AGC CGT CGG TGG TTA CTT GAG TAG GGC AG	2.10 µg/µL
VNTR-8	**NED**·GTG GGA AGC GTT ATC CTT TAA CGG GAG TAA GGG	NA[b]
VNTR-9	**HEX**·GGA AAT CCG CAT CGT GGC CTT CG	1.55 µg/µL
VNTR-10	**NED**·GCG GAG GGC GAC AAG GCG AAC	NA[b]
REV-1	GGG GAG TAT GTT TTG GTT GCG CAT GAC CGC	1.87 µg/µL
REV-3	GGG GGC ART ARG GCA GTA TGT TAA GGG AAT AGG G	2.15 µg/µL
REV-9	GAT CTG CTT CGG ATA GGC GCG GCG TGA G	1.57 µg/µL
REV-10	GGT GCG GGC AGG TGG TGG ACA AGG C	1.74 µg/µL

[a] 1000X has a concentration of 200 mM (*see* **Note 19**).

[b] NED is a proprietary dye of Applied Biosystems and its contribution to the molecular weight of the labeled oligo is not available. However, the custom synthesis of a primer containing NED is shipped with a report of the total number of pmol synthesized, from which the resuspension volume to make a 200 pmol/µL stock solution can be calculated.

Table 2
Primer Cocktails

Primer Cocktail 1		Primer Cocktail 2		Primer Cocktail 3		Primer Cocktail 4	
Primer[a]	Amount	Primer	Amount	Primer	Amount	Primer	Amount
VNTR-1	1 µL	VNTR-4	1 µL	VNTR-5	1 µL	VNTR-2	1 µL
VNTR-3	1 µL	VNTR-6	1 µL	VNTR-10	1 µL	VNTR-7	1 µL
Rev-3	2 µL	VNTR-9	1 µL	Rev-3	1 µL	VNTR-8	1 µL
		Rev-3	2 µL	Rev-10	1 µL	Rev-1	3 µL
		Rev-9	1 µL				
Water	96 µL	Water	94 µL	Water	96 µL	Water	94 µL

Primer Cocktail 5		Primer Cocktail 6		Primer Cocktail 7		Primer Cocktail 8	
Primer	Amount	Primer	Amount	Primer	Amount	Primer	Amount
VNTR-1	1 µL	VNTR-2	1 µL	VNTR-3	1 µL	VNTR-7	1 µL
VNTR-4	1 µL	VNTR-10	1 µL	VNTR-5	1 µL	VNTR-9	1 µL
Rev-3	2 µL	Rev-1	1 µL	Rev-3	2 µL	Rev-1	1 µL
		Rev-10	1 µL			Rev-9	1 µL
Water	96 µL	Water	96 µL	Water	96 µL	Water	96 µL

[a] *See* **Notes 5, 20,** and **21**.

Table 3
Master Mix Formulations
(Prepare Eight Sets of the Formulation Listed Below, One for Each Primer Cocktail)

Component	1X (per well)	13X (per plate)
Water	9.7 µL	126.2 µL
10X Buffer [a]	2.8 µL	36.4 µL
2.5 m*M* dNTP mix [b]	2.5 µL	32.5 µL
Primer cocktail mix [c]	2.5 µL	32.5 µL
25 m*M* $MgCl_2$ Stock solution	1.7 µL	22.1 µL
GC Rich Enhancer [d] 5X conc.	5.6 µL	72.8 µL
FastStart Taq DNA polymerase [e] (5 U/µL)	0.2 µL	2.5 µL
Total vol.	25 µL	325 µL

[a] 500 m*M* Tris-HCl, 100 m*M* KCl, 50 m*M* $(NH_4)_2SO_4$, pH 8.3 @ 25°C. Included with FastStart Taq DNA polymerase.
[b] 2.5 m*M* each of dATP, dCTP, dGTP, dTTP.
[c] From **Table 2**.
[d] GC Rich Enhancer is a proprietary formulation included with Fast Start Taq Polymerase. Its use is optional. We see a slight increase in PCR specificity when it is included in the reaction mix. If this solution is not used in the reaction mix, an equivalent volume of water must be substituted.
[e] *See* **Note 6**.

2.5. Sizing of Amplicons by Capillary Electrophoresis

1. Capillary electrophoresis instrument with fluorescent fragment detector, suitable for small DNA fragment analysis (e.g., ABI Prism 3100 Genetic Analyzer, Applied Biosystems).
2. Deionized formamide (Applied Biosystems; *see* **Note 15**).
3. CE running buffer (AMRESCO Inc.) or 10X Genetic Analyzer Buffer with EDTA (Applied Biosystems).
4. Fluorescent internal sizing ladder range 50 to 350 bp (Applied Biosystems).
5. Capillary matrix polymer for fragment separation (Applied Biosystems).
6. Data capture software (Applied Biosystems).
7. 36-cm Capillary array (16 capillaries/array; Applied Biosystems).
8. Computer for data analysis.

3. Methods

3.1. PCR Setup

The extreme sensitivity of PCR makes this technique highly vulnerable to contamination artifacts. Because a single copy of template can be amplified into significant yields of corresponding products, significant care and planning must be implemented before performing any PCR assays for diagnostic purposes.

Preparation of bulk quantities of the reaction mixture should be done in a dedicated room, biological safety cabinet, or enclosed work station that is free of bacteria or DNA.

It is not sufficient to simply wipe down the work area areas with alcohol or certain bactericidal solutions because the DNA of the dead bacteria can still be amplified by PCR. Work surfaces and equipment must be treated with a decontaminant shown to destroy DNA. For example, treatment of surfaces and equipment with 10% bleach ***(20)*** will destroy the DNA as well as kill the bacteria as long as all surfaces are exposed. Ultraviolet (UV) radiation also will destroy DNA and is a convenient way to decontaminate biological cabinets or enclosed workstations. Pipets, tube racks, and stock solutions should be dedicated to PCR. Addition of the sample template, amplification, and detection should be performed in another area with a separate set of equipment and supplies than those used for making the master mix. The use of disposable pipet tips containing aerosol-preventing filters is highly recommended for all stages of the procedure.

3.2. Sample Preparation

The DNA used for strain typing comes from the bacteria cultured from infected tissues or exudates as part of the normal diagnostic process (*see* **Note 16**). All HOOF-print analyses should be performed on individual colonies either directly or subcultured for archiving. Because of the potential for microevolution within the bacterial population, it is highly recommended that several colonies (5–20) are individually tested for each strain.

1. Cultivate *B. abortus* from abortion material, lymph nodes, milk, and reproductive organs on a primary isolation plate (a basic nutrient-agar plate, such as tryptose agar, trypticase-soy agar, or Brucella agar, containing 5% serum and antibiotics ***[4]***). Incubate in a 10% CO_2 atmosphere at 37°C for 24–72 h.
2. If pure colonies of *Brucella* cannot be obtained from the primary isolation plate, reinoculate suspected *Brucella* colonies onto a secondary isolation plate and incubate at 37°C in a 10% CO_2 atmosphere for 24 to 48 h.
3. Culture suspensions may be preserved in methanol for later use and archiving (*see* **Note 3**). Using a sterile inoculation loop, transfer bacteria from a single colony to a flask containing 30 mL of trypticase soy broth with 5% bovine serum. Incubate in a shaking 37°C water bath for 24 to 72 h until a heavy growth is achieved. Transfer the bacteria/broth suspension to a screw-top centrifuge tube that has been weighed. Centrifuge the sample at 7000*g* for 15 min. Discard the supernatant into a bactericidal disinfectant (e.g., 1% Lysol IC) and determine the wet-weight of the pellet. Resuspend the pellet in 100 µL of methanol and 50 µL of saline for each mg wet-weight of the pellet. Incubate the sample at 4°C for at least a week, thoroughly mixing by inversion daily, to ensure complete killing of the bacteria. Samples can be conveniently stored at 4°C in 20-mL scintillation-type vials with screw caps for at least 5 yr without significant degradation or loss of DNA.

4. To prepare bacteria directly from agar plates (primary or secondary) for PCR, use a sterile inoculating loop to transfer bacteria from one large colony to a 2-mL tube containing 100 µL of saline.
5. Before use, remix the culture suspension or methanol preserved suspension and dilute an aliquot 1/10 in PCR-grade water. Mix gently but thoroughly. The diluted material should be appropriately discarded after use (*see* **Note 17**).

3.3. PCR Amplification

3.3.1. Preparation of Multiplex Master Mixes

Each sample is characterized with eight multiplexed PCR primer combinations, in which most HOOF-print loci are tested twice for reproducibility (*see* **Note 18**). A total of 10 unknown samples along with 1 positive control sample and 1 negative control sample can be analyzed in a 96-well PCR plate format.

1. Prepare a 1000X stock of synthesized oligonucleotide primers in TE buffer (*see* **Notes 8** and **19**).
2. Prepare each of the eight primer-cocktail working solutions according to the protocol in **Table 2** (*see* **Notes 5, 20**, and **21**). The diluted working solution can be stored in the dark at 4°C for approx 3 d without deterioration.
3. Prepare eight PCR Master Mixes (one for each primer combination) as outlined in **Table 3**. Add each component sequentially. Mix completely after the addition of GC Rich solution and again after the addition of the polymerase (*see* **Note 22**).

3.3.2. PCR Setup

1. Following the diagram in **Fig. 1**, aliquot 25 µL of Master Mix into the bottom of each well across one entire row. This can easily be done with a positive displacement repeating pipettor.
2. If it will be more than a few minutes until the sample templates are ready to add, cover the plate with an adhesive plate sealer to prevent evaporation and store the plate at 4°C (up to 24 h).
3. When the template samples are ready, remove the plate from 4°C and examine the wells from beneath the plate to see that all wells are filled and that none of the liquid remains on the sides of the well or condensed onto the coversheet. If some droplets of solution are on the sides or tops of the wells, tap the plate gently on the lab bench or pulse briefly in a centrifuge equipped with a plate rotor to collect all the fluid at the bottom. Always be sure that the wells are tightly sealed before tapping or centrifuging the plate to prevent cross-contamination.
4. Add 3 µL of one diluted bacterial strain to each of the eight wells in a column (*see* **Fig. 1**). Fill the first column with a positive control strain or an allelic ladder and then fill the last column with PCR-grade water as the negative control. Be certain that the sample is deposited into the solution at the bottom of the well. The same tip can be used for the entire column if the tip is wiped with a tissue after each addition to remove any liquid adhering to the outside of the tip (*see* **Note 23**).

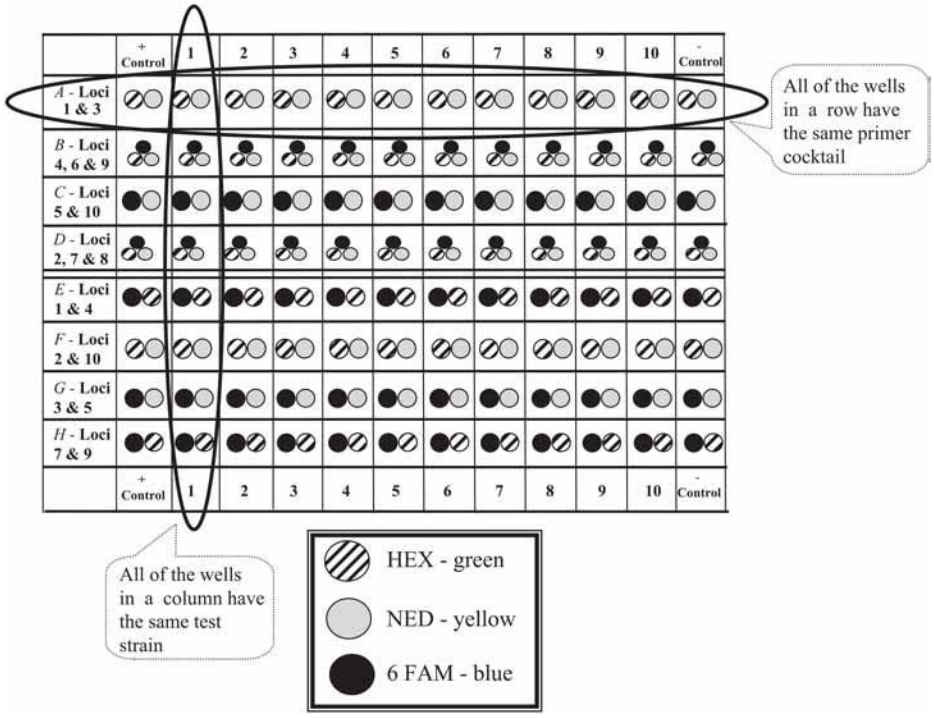


Fig. 1. Layout of the hypervariable octameic oligonucleotide fingerprints (i.e., HOOF print) assay in a 96-well polymerase chain reaction (PCR) plate format. Samples for PCR are arranged so that each row contains a multiplex Master Mix for amplification of two or three loci. One strain is added to each column. The color combination of dyes is indicated by patterned circles in each well.

5. Cover the plate with cap strips (*see* **Note 10**), a cap mat, or a fresh adhesive backed sheet of aluminum foil or plastic PCR film (*see* **Note 11**). The use of a plate roller is recommended to be certain that each well is completely sealed.
6. The following parameters are used to amplify the target DNA (*see* **Notes 24–26**): 95°C for 5 min followed by 30 cycles of 95°C for 30 s, 53°C for 30 s, 75°C for 30 s, and one cycle of 75°C for 60 min.

3.4. Processing of Amplicons: Monitoring Amplification Success

1. Prepare two 48-well (double decked) 4% agarose gels. Remove residual buffer from the wells. Be sure that the outside surface of the gel cassette is clean and free of liquid. For electrophoresis in a system that uses a running buffer, see **Note 27**.

2. Load each well with 5 µL of amplified sample in a total volume of 20 µL. The easiest way to do this is to use an expandable 12-channel programmable pipettor with the following program: pick up 17 µL (water); pick up 5 µL (sample); dispense 20 µL (into gel well); purge. No loading buffer or tracking dye is needed with a bufferless system (*see* **Notes 28**–**30**).
3. In the remaining marker lanes, add a 10-bp DNA ladder and a 25-bp DNA ladder at 250 to 500 ng per lane. For double-decked gels, be sure to include markers in each row of samples.
4. Place the gel cassette in the electrophoresis apparatus and connect the system to the power supply. Run the gels at 70 V for 20 to 30 min at room temperature.
5. If the gel has not been prestained with ethidium bromide, then the gel should be soaked for 10 min in a solution of 250 µg of ethidium bromide in 500 mL of the running buffer, or for greater sensitivity in SYBR Gold Nucleic Acid Gel Stain. If the background fluorescence is too bright under UV illumination, the gel can be destained in distilled water for 20 min (*see* **Notes 13** and **14**).
6. Record the gel image digitally or on film under UV illumination (312 nm). Examples of typical singleplex and multiplex results are shown in **Fig. 2.**

3.5. Sizing of Amplicons by Capillary Electrophoresis With Fluorescent Detection

Identification of alleles that differ by 8 bp requires a size analysis method that is capable of very precise measurements. Capillary electrophoresis ***(21)*** with fluorescent detection has this capability. The high sensitivity allows minute amounts of sample to be sharply resolved by differential migration through a 36-cm electric field.

3.5.1. Sample Preparation

Samples should be diluted in PCR-grade water, typically in the range of 1:4 through 1:200, depending on the amplicon concentration, which can be estimated from the intensity of the DNA staining displayed on the agarose gel. If the product band is very light or not visible, a 1:4 dilution is an appropriate starting point. The diluted samples are then prepared for electrokinetic injection into the capillary array by mixing 1.5 µL of the diluted PCR product with 0.25 µL of the internal size standard (e.g. GeneScan-500 [ROX]) and 9.75 µL of deionized formamide (*see* **Note 15**).

3.5.2. Controls

In addition to a PCR-positive control strain, size determination by capillary electrophoresis is more accurate with a set of allelic ladders or amplicon standards for each primer cocktail (*see* **Subheading 3.7.**). An allelic ladder can be prepared by pooling a number of individually amplified alleles (*see* **Fig. 2**) in equalized amounts that span the typical range of allelic sizes at that locus (*see* **Note 31**). Amplicon standards are made by pooling the individually amplified

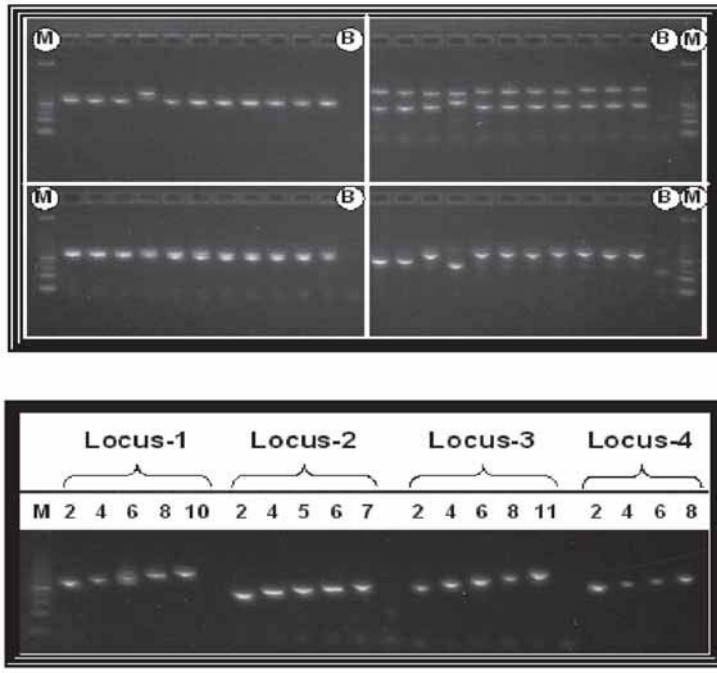


Fig. 2. Analysis of polymerase chain reaction amplicons by electrophoretic separation through a 4% agarose gel (48-well E-gel). Top: each quadrant of the gel contains the multiplex amplicons of one primer combination tested on 10 *Brucella abortus*. Notice that all two or three bands may not be resolved in a short running distance (e.g., 3.2-cm for the 48-well E-gels). M = 25-bp ladder (molecular size marker), B = blank (negative control). Bottom: singleplex allelic ladders from four loci. The stair-step patterns visibly demonstrate the discrimination of individual alleles over a short migration distance. M = 25-bp ladder (molecular size marker); the number above each lane indicates the allele (number of repeat units) amplified. Amplicons were detected by ultraviolet illumination in the presence of ethidium bromide.

alleles from a few, well-characterized strains, in which the individual alleles have been confirmed by DNA sequencing. In this case, the allelic sizes will not be a regularly spaced ladder, but they are easier to prepare than a true ladder.

3.5.3. Capillary Electrophoresis Conditions

The following conditions apply to the ABI Prism 3100 Genetic Analyzer.
Run information:

- Data collection version: 1.0.1
- Analysis Module: GS500 Analysis.gsp
- Run Module: GeneScan36_POP4DefaultModule

Data collection settings:

- Prerun Voltage: 15 kV
- Injection Voltage: 1 kV
- Run Voltage: 15 kV
- Temperature: 60°C
- Prerun time: 3 min
- Injection Duration: 22 s
- Run Time: 25 min

Gel information:

- Capillary separating polymer gel matrix: POP4
- Capillary length: 36 cm
- Run buffer: A.C.E CE Running Buffer - 1X concentration

3.6. Data Analysis

The capillary electrophoresis systems with fluorescent detection all use complementary software packages for data capture and analysis. A detailed review of these software packages is beyond the scope of this chapter. However, some familiarity with the data output will be helpful for the interpretation of results. The following briefly describes the basics for using GeneScan software to view and record data from the ABI Prism 3100 Genetic Analyzer.

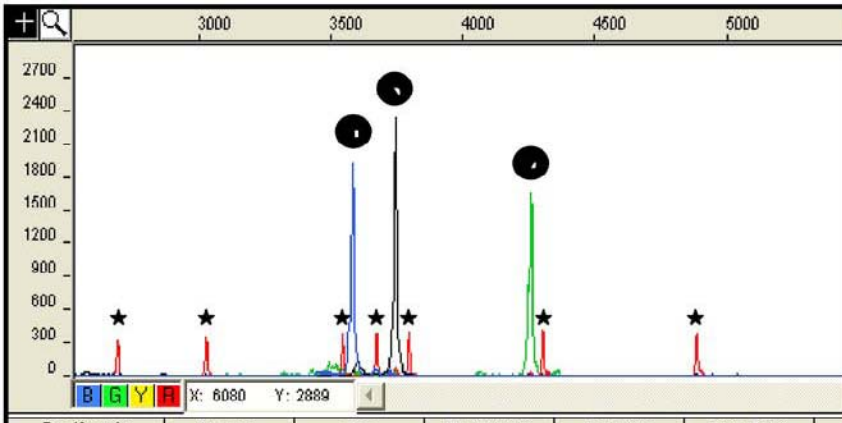
3.6.1. Creating a Project

1. If the data have been saved as a zip or a tar file to conserve space, the file must be extracted with an appropriate program (e.g., WinZip, WinZip Computing Inc).
2. Open the GeneScan software program.
3. View the menu options under the **File** tab on the main toolbar; highlight the **<New>** option. A pop-up menu will appear with several icons. Single-click the **Project** icon.
4. The Analysis Window will appear.
5. View the menu options under the **Project** tab on the main toolbar of the Analysis Window. Highlight the **<Add sample files>** option. Browse through the directories by double-clicking the appropriate folder icons along the path to the extracted data files (the file names will have an "fsa" extension). Each file contains data for 1 of the 96 samples that were analyzed. Highlight individual files for loading and press the **<Add>** button. Alternatively, if all the files will be viewed simply press the **<Add All>** button. The files to be loaded will appear in the second window at the bottom of the screen. If the files listed in this box are the correct files to be viewed, press the **<Finish>** button.
6. The list of individual files now appears in the Analysis window.

3.6.2. Viewing and Recording the Data

1. Double click one of the Sample File cells to open a new window containing the electropherogram and a corresponding data table (*see* **Fig. 3**).

Fig. 3. *(Opposite page)* Example of a GeneScan screen displaying the electropherogram and data table for the multiplexed polymerase chain reaction products from three loci. Normally shown in color, the electropherogram window displays peaks that indicate a specific dye was detected by one of the four color sensors. *(Continued)*

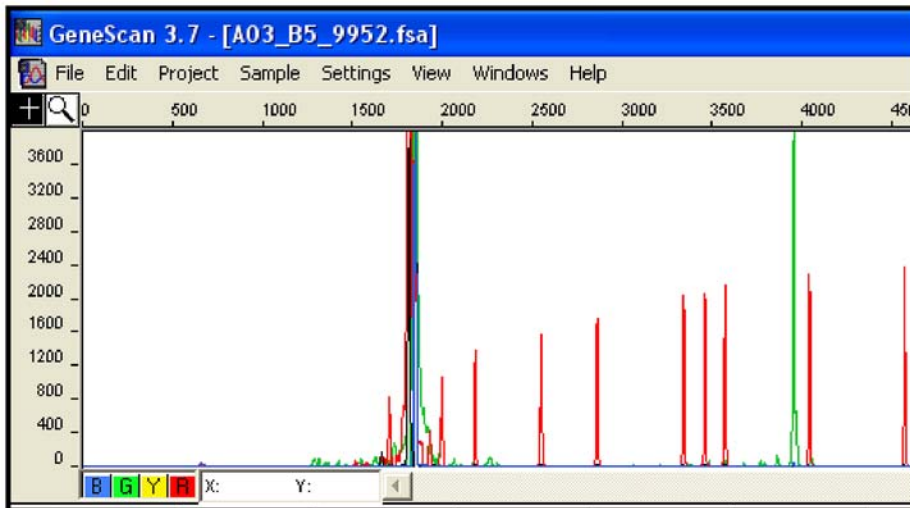


Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
B, 1	9.55	142.27	1985	30833	3581
B, 2	9.78	149.74	82	1737	3668
B, 3	9.93	154.19	54	543	3722
G, 1	9.14	129.77	76	1005	3426
G, 2	9.28	133.92	65	565	3479
G, 3	9.31	134.87	145	1613	3491
G, 4	9.34	135.90	118	1234	3504
G, 5	9.38	136.93	126	1431	3517
G, 6	9.41	137.88	78	648	3529
G, 7	9.47	139.60	82	1442	3550
G, 8	9.98	155.81	54	667	3742
G, 9	11.35	196.47	1705	26951	4256
G, 10	11.39	197.72	144	1585	4272
G, 11	11.62	204.52	58	567	4358
Y, 1	9.60	143.89	145	4193	3601
Y, 2	9.98	155.89	2420	35532	3743
R, 1	7.18	75.00	338	3397	2692
R, 2	8.07	100.00	376	3629	3027
R, 3	9.45	139.00	401	3986	3543
R, 4	9.79	150.00	410	4000	3671
R, 5	9.98	155.81	84	1576	3742
R, 6	10.12	160.00	417	4012	3794
R, 7	11.47	200.00	429	4327	4301
R, 8	13.02	246.91	394	5206	4883
R, 9	14.71	300.00	455	5613	5516
R, 10	15.02	340.00	430	6746	6070
R, 11	16.26	350.00	466	5975	6097
R, 12	17.93	400.00	497	6584	6686
R, 13	19.31	450.00	477	6809	7242
R, 14	20.49	490.00	469	7260	7684
R, 15	20.75	500.00	469	7320	7792

Peak sizes

Fig. 3. *(Continued)* In this example, the red peaks (corresponding to the internal size markers) are marked with stars; the blue peak is labeled "B," the black/yellow peak is labeled "Y," and the green peak is labeled "G". The *x*-axis is the migration time through the capillary (from the injector to the detector), whereas the *y*-axis is the relative fluorescent units detected by the sensor. In the data table, the migration times are converted to molecular sizes (in base pairs) calculated from the calibration curve that is created for every sample from the internal size markers.

2. The electropherogram consists of a series of peaks along the *x*-axis. The heights of the peaks (*y*-axis) are measured in relative fluorescent units (RFUs) that were emitted as the DNA fragments passed by the detector. The color of the peak corresponds to the dye color detected in the sample except that black is substituted for the yellow dye (NED) for better visibility. With the HOOF-print multiplex protocol, either three or four colors will appear in each electropherogram. All samples contain a series of red peaks (marked with a star above the peak in **Fig. 3**). These peaks are from the ROX-500 internal size standard used to calibrate the size calling curve. The other peaks identify the alleles amplified with the primer cocktail used with the sample.
3. The results table (*see* **Fig. 3**) lists the specific values for the data shown in the electropherogram. The table provides the following information:
   a. *Dye/Sample Peak:* This column identifies the dye color, and sequentially numbers all of the fragments that fluoresce above the threshold limit (e.g., 50 RFU) set to distinguish peaks from background noise.
   b. *Minutes:* This displays the time required for each fragment to migrate through the capillary to the detector.
   c. *Size:* This is the size of the fragment in base pairs calculated from the size curve generated by the internal standards.
   d. *Peak height:* This is the maximum number of fluorescent units recorded when the fragment passed by the detector.
   e. *Peak area:* This is the total calculated fluorescent units emitted by the fragment as it passed by the detector.
   f. *Data point:* This column identifies the position of the peak along the *x*-axis of the electropherogram.
4. Another notable feature of the electropherograms is the presence of a large peak near the start (left edge) of the chromatogram (*see* **Fig. 4**). This peak contains an assortment of small-sized DNA fragments that run directly through the capillary, relatively unhindered by the gel matrix. The run-through peak is composed mainly of primers and primer-dimers and should be ignored. The data analysis software can be programmed so that the fragment size-threshold for the data table is larger than the sizes of the run-through fragments. As a result, the run-through peak will not be displayed in the table. It is not necessary to record all the data in the data table. The data can be adequately summarized by recording the fragment size in base pairs and the peak height. The peak height is a useful measure of the relative abundance of the fragment. If the peak height is very small (less than 100 units) the corresponding peak might not be significantly greater than the background fluorescence (*see* **Note 32**). If the concentration of the allele fragment is too high (e.g., greater than 7000 RFU), the emitted light can overload the sensors, cause overestimation of background products and contaminants, or cause other problems. Emissions in the range of 1000 to 4000 RFU generally produce unambiguous results. A convenient table layout for recording data can be formatted with a spreadsheet program. An example of an Excel-type spreadsheet layout is given in **Fig. 5**. Since the spreadsheet contains data for 228 individual alleles, the spreadsheet template can be set up with color-coded fonts to help simplify the data, visually.



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
B, 1	10.53	192.53	72	719	3948
G, 1	5.50	40.94	108	1051	2063
G, 2	5.76	48.77	53	362	2161
G, 3	6.03	55.79	126	3196	2261
G, 4	6.13	58.45	57	678	2300
G, 5	9.27	151.98	74	667	3477
G, 6	9.53	160.68	68	501	3575
G, 7	9.78	168.60	56	538	3668
G, 8	10.03	176.62	81	692	3762
G, 9	10.09	178.33	68	1094	3782
G, 10	10.28	184.65	154	1708	3856
G, 11	10.53	192.70	4550	44963	3950
G, 12	10.57	193.73	687	4565	3962
G, 13	10.78	200.66	224	2180	4042
Y, 1	13.72	299.83	53	558	5145

Fig. 4. Example of a GeneScan screen displaying the electropherogram and data table for the polymerase chain reaction (PCR) products from one locus. In this example, the electropherogram shows the PCR products as single sharp peak among the internal size markers' peaks. A large peak that elutes very early from the capillary (labeled the run-through peak) contains very small deoxyribonucleic acid fragments such as primers and primer-dimers. Although not obvious in the electropherogram, the sample peak and the plus-A peak are recorded by the software in the data table (circled in the table). Stars mark the peaks formed by the internal size markers.

3.7. Interpretation of Results

HOOF-print genotypes consist of the specific alleles identified from 10 polymorphic genomic loci. Alleles are named for the calculated number of complete repeat units at each locus because each locus also contains a characteristic partial repeat in addition to the complete repeat units. Using Locus-1 as an

DATE
Project No.
Plate No.
Positive Control
Sample-1
Sample-2
Sample-3
Sample-4
Sample-5
Sample-6
Sample-7
Sample-8
Sample-9
Sample-10
Negative Control (Blank)
Sample-1
Locus-1 Fragme
Locus-3 Fragme
162.12
1865
140.42
2460
L1 - L3
Alleles
DATE
Project No.
Plate No.

Fig. 5

example, Allele 2 has 2 and 3/8 repeat units, Allele 3 has 3 and 3/8 repeat units; and so on. The sizes of the respective partial repeats for the 10 loci are:

Locus-1	3/8
Locus-2	1/8
Locus-3	6/8
Locus-4	1/8
Locus-4	1/8
Locus-5	1/8
Locus-6	3/8
Locus-7	3/8
Locus-8	3/8
Locus-9	1/8 + (–1/8)
Locus-10	(–1/8)

Locus-9 contains two series of 8-bp repeats that are different in sequence, are approx 40 bp apart and appear to mutate independently. When choosing the amplification primers for this locus, two primer sets were tested, and it was discovered that the best performing primers amplified both groups of tandem repeats. One of the tandem repeat groups has one extra base pair of the repeat sequence (+1/8), whereas the other series lacks one base pair of the final repeat unit (–1/8). Therefore, Allele 4 may actually consist of 2 and 1/8 repeat units, plus 1 and 7/8 repeat units. Locus 10 also contains a partial final repeat unit that is one base pair short of complete, so that Allele 2 is actually 1 and 7/8 repeat units.

A conversion table for determining the number of repeats at each locus based on the fragment size is presented in **Table 4**. This table lists the *theoretical* molecular size in base-pairs calculated from DNA sequence analysis of multiple alleles for each locus. However, in practice, the observed fragment sizes determined by capillary electrophoresis were found to differ from the predicted molecular sizes by as much as 5 to 6 bp. Fortunately, with proper controls and consistent assay conditions, these variations arise in predictable patterns that permit reproducible allele assignments.

A number of reasons exist for the discrepancies between the theoretical allele sizes and the observed sizes. It has been shown that the mobilities of rhodamine-based dyes (ROX and TAMRA) in POP4 polymer-filled capillaries are not consistent with the mobilities of fluorescein dyes (i.e., 6-FAM, HEX, NED, JOE, and TET). The differences can range as high as 6.5-bp for a 100-bp fragment ***(22)***.

Fig. 5. *(Opposite page)* Example of an Excel spreadsheet design useful for summarizing capillary electrophoresis data and resulting allele assignments. The table layout is based on the layout of the corresponding polymerase chain reaction plate. The table contains three types of data for each sample: fragment size, peak height, and allele assignment. Loci are abbreviated “L” (e.g., L1 for Locus-1).

Table 4
Allele Assignment Based on Theoretical Amplicon Size (in bp)

Allele (no.)	VNTR-1	VNTR-2	VNTR-3	VNTR-4 [a]	VNTR-5	Allele (no.)	VNTR-6	VNTR-7	VNTR-8	VNTR-9	VNTR-10	Allele (no.)
M	124 bp	na	na	na	na	M	142 bp	na	na	na	na	M
1	132 bp	93 bp	127 bp	131 bp	130 bp	1	150 bp	79 bp	83 bp	125 bp	201 bp	1
2	140 bp	101 bp	135 bp	139 bp	138 bp	2	158 bp	87 bp	91 bp	133 bp	212 bp	2
3	148 bp	109 bp	143 bp	147 bp	146 bp	3	166 bp	95 bp	99 bp	141 bp	220 bp	3
4	156 bp	117 bp	151 bp	155 bp	154 bp	4	174 bp	103 bp	107 bp	149 bp	228 bp	4
5	164 bp	125 bp	159 bp	163 bp	162 bp	5	182 bp	111 bp	115 bp	157 bp	236 bp	5
6	172 bp	133 bp	167 bp	171 bp	170 bp	6	190 bp	119 bp	123 bp	165 bp	244 bp	6
7	180 bp	141 bp	175 bp	179 bp	178 bp	7	198 bp	127 bp	131 bp	173 bp	252 bp	7
8	188 bp	149 bp	183 bp	187 bp	186 bp	8	206 bp	135 bp	139 bp	181 bp	260 bp	8
9	196 bp	157 bp	191 bp	195 bp	194 bp	9	214 bp	143 bp	147 bp	189 bp	268 bp	9
10	204 bp	165 bp	199 bp	203 bp	202 bp	10	222 bp	151 bp	155 bp	197 bp	276 bp	10
11	212 bp	173 bp	207 bp	211 bp	210 bp	11	230 bp	159 bp	163 bp	205 bp	284 bp	11
12	220 bp	181 bp	215 bp	219 bp	218 bp	12	238 bp	167 bp	171 bp	213 bp	292 bp	12
13	228 bp	189 bp	223 bp	227 bp	226 bp	13	246 bp	175 bp	179 bp	221 bp	300 bp	13
14	236 bp	197 bp	231 bp	235 bp	234 bp	14	254 bp	183 bp	187-b[	229 bp	308 bp	14
15	244 bp	205 bp	239 bp	243 bp	242 bp	15	262 bp	191 bp	195 bp	237 bp	316 bp	15
16	252 bp	213 bp	247 bp	251 bp	250 bp	16	270 bp	199 bp	203 bp	245 bp	324 bp	16
17	260 bp	221 bp	255 bp	259 bp	258 bp	17	278 bp	207 bp	211 bp	253 bp	332 bp	17
18	268 bp	229 bp	263 bp	267 bp	266 bp	18	286 bp	215 bp	219 bp	261 bp	340 bp	18
19	276 bp	237 bp	271 bp	275 bp	274 bp	19	294 bp	223 bp	227 bp	269 bp	348 bp	19
20	284 bp	245 bp	279 bp	283 bp	282 bp	20	302 bp	231 bp	235 bp	277 bp	356 bp	20
21	292 bp	253 bp	287 bp	291 bp	290 bp	21	310 bp	239 bp	243 bp	285 bp	364 bp	21
22	300 bp	261 bp	295 bp	299 bp	298 bp	22	318 bp	247 bp	251 bp	293 bp	372 bp	22
						23	326 bp	255 bp	259 bp	301 bp	380 bp	23
						24	334 bp	263 bp	267 bp	309 bp	388 bp	24
						25	342 bp	271 bp	275 bp	317 bp	396 bp	25

Na, not available. [a]Allele sizes for Locus-4 apply only to *B. abortus* and *B. melitensis* strains. For all other *Brucella* species, refer to **Table 5**.

The HOOF-print genotypes are written as a sequential numerical string of ordered alleles calculated for Loci 1 to 10. For example, the HOOF print for the vaccine strain, *B. abortus* S19 is: 5, 4, 4, 2, 2, 2, 8, 2, 13, 6, meaning that Locus-1 has Allele 5; Locus-2 has Allele 4; Locus-3 has Allele 4; and so on. This format makes it very easy to construct a multilocus DNA fingerprint database that could be used by any laboratory in the world. The development of an International *Brucella* Fingerprint Database is currently in progress.

3.8. Troubleshooting Potential Problems With the Experimental Data

There are a number of things that can affect data production or data interpretation. The following section describes some of the more common problems encountered when performing the HOOF-print assay. This section also shows that it is essential to carefully examine the data before reaching a final determination. One particular danger is the overreliance on allele-calling software that automatically assigns the data into bins (alleles) based on size. These programs may not be able to distinguish between true and false signals and, therefore, it is important to visually inspect and confirm allele identifications.

3.8.1. Abnormal Migration of Locus-4 Alleles

It was noticed early in the development of the HOOF-print assay that *B. suis* isolates exhibit abnormal migration behavior for Locus-4 alleles. Sequence analysis of selected alleles showed that *B. suis* isolates contain a specific mutation in the conserved flanking DNA sequence that increases the size of each allele by 11-bp ***(19)***. Because of the 11-bp change, the *B. suis* alleles for Locus-4 do not match the theoretical allele sizes for *B. abortus* and *B. melitensis* strains. An alternative allele size chart for Locus-4 is shown in **Table 5**. Alleles listed on the alternative chart are designated with an "A" (e.g. Allele 3A). Further studies have shown that *B. canis*, *B. ovis*, *B. neotomae*, *B. cetaceae*, and *B. pinnipediae* strains follow the *B. suis* allele size predictions.

3.8.2. Double Peaks Caused by Nontemplated Nucleotides

DNA polymerases (e.g., Taq DNA polymerase) that lack 5′ to 3′ exonuclease activity will add an extra nontemplated nucleotide (typically dATP) to amplified products. However, the addition of the nontemplated base is not absolute; therefore, amplifications performed with a Taq-type polymerase will consist of two populations of amplicons: those with the nontemplated base (sometimes referred to as "Plus-A") and those without the extra base. During analysis, these populations will be resolved into a double peak separated by 1 bp. Often, the double peak is not resolved clearly in the electropherogram but instead is listed in the data table as two peaks (*see* **Fig. 4**).

Table 5
Alternate Allele Assignment for Locus-4 Based on Amplicon Size (bp)

Allele (no.)	VNTR-4	Allele (no.)
1A	142 bp	1A
2A	150 bp	2A
3A	158 bp	3A
4A	166 bp	4A
5A	174 bp	5A
6A	182 bp	6A
7A	190 bp	7A
8A	198 bp	8A
9A	206 bp	9A
10A	214 bp	10A
11A	222 bp	11A
12A	230 bp	12A

This table should be used for the following *Brucella* species: *B. canis*, *B. neotomae*, *B. ovis*, *B. suis*, *B. cetaceae*, and *B. pinnipediae*.

During PCR, the 60-min, 75°C incubation, inserted after the last amplification cycle, drives the process toward maximal addition of the nontemplated nucleotide, as demonstrated in **Fig. 6**. However, the nontemplated addition is influenced by the flanking sequence so that some loci produce mainly the "true" allele product whereas other loci produce mostly the "plus-A" product. Nontemplated nucleotide addition is rarely a problem for identifying HOOF-print alleles because allele sizes differ by 8-bp units. However, this is one factor that causes the observed amplicon size to differ from the theoretically calculated size based sequence data.

3.8.3. Stutter Peaks

Stutter peaks are caused by imperfect amplicons containing too many or too few repeat units. This phenomenon is caused by slip-strand mispairing during PCR amplification, whereby the DNA polymerase essentially loses its place when copying a large tract of repeats. These amplicons differ from the original DNA template in multiples of 8 bp. Although all variable number tandem repeat (VNTR) loci will produce stutter products during amplification, the proportion of stutter products decreases as the length of the repeat sequence increases. Thus, loci containing 2-bp repeat units have the highest proportion of stutter products.

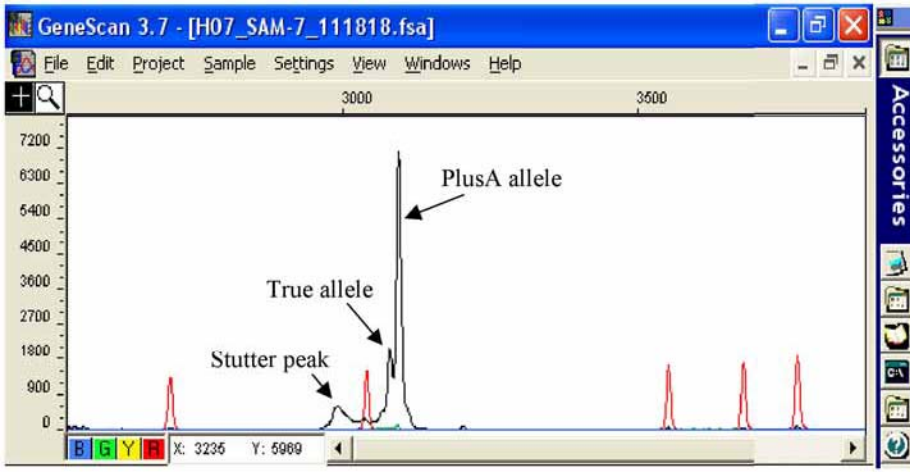


Fig. 6. Example of an electropherogram showing the deoxyribonucleic acid doublet composed of from the true allele and the Plus-A allele that is formed by the terminal transferase activity of the Taq DNA polymerase. In this example, the majority of the amplicons are in the Plus-A form. Stars mark the peaks formed by the internal size markers.

The size of the repeat string also affects the amount of stutter produced. Long strings have a greater chance of slippage than short strings.

Typically, stutter products are of minor concern for HOOF-print analyses because an 8-bp repeat size is not particularly prone to slippage (*see* **Figs. 3** and **4**). However, for loci that contain a large number of repeats, or if the assay is performed under nonideal conditions, stutter peaks can be significant and may even obscure alleles from other loci. **Figure 7** shows an example of significant stutter. **Figure 7A** shows the complex results from a multiplexed combination of primers for 3 loci (Loci-2, -7, and -8). The straight arrows point to the stutter peaks and the associated true peak for Locus-7. As shown in the corresponding table, the stutter peaks differ in 8-bp intervals (data indicated with a single heavy circle). The enlarged insert (**Fig. 7B**) shows the stutter peaks more clearly after subtracting the data for Loci-2 and -8. Notice that the stutter radiates out in both directions from the true peak but that the stutter peaks on the negative side (loss of repeat units) are higher (i.e., more DNA) than the peaks on the positive side (addition of repeat units). Also, note that each stutter peak becomes progressively smaller in height (less DNA), the further they are from the true peak.

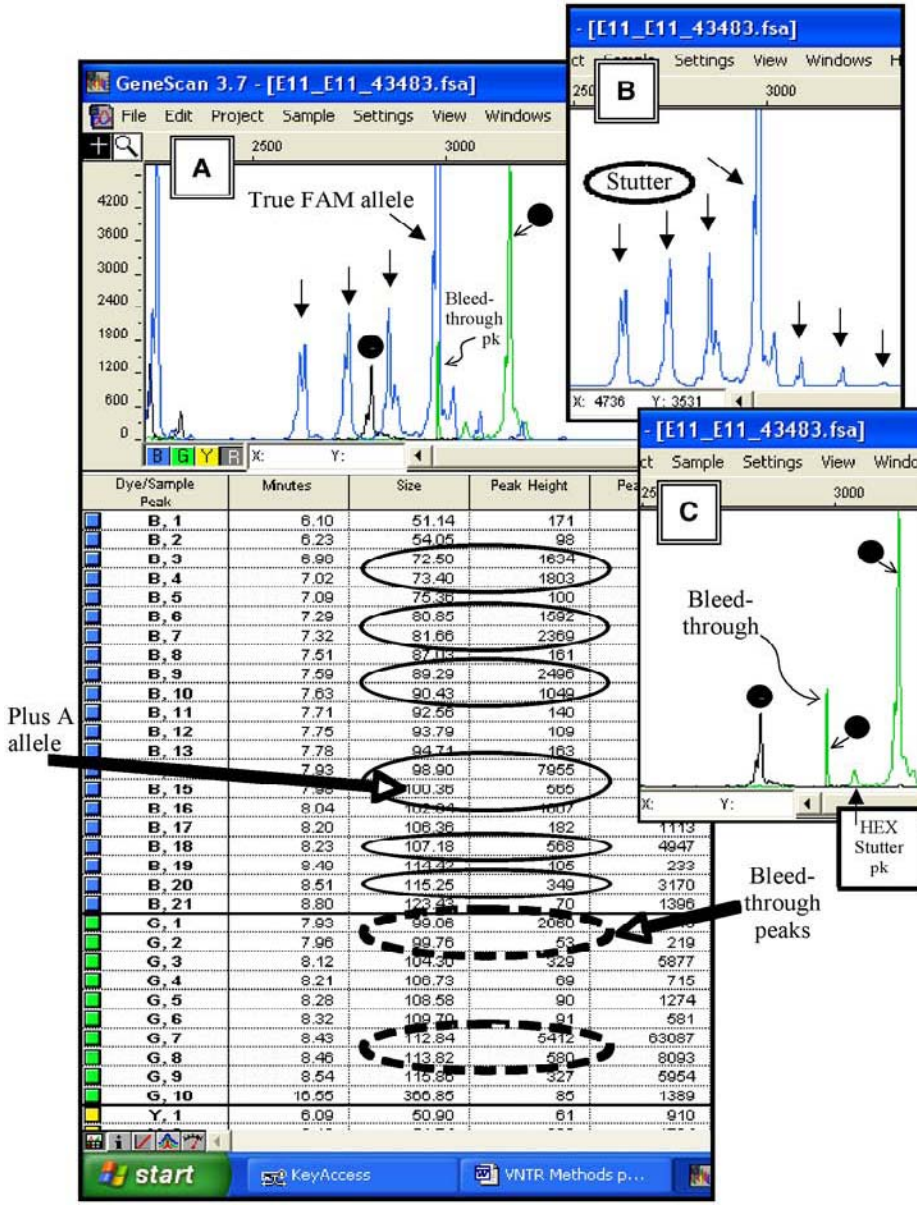


Fig. 7. Problems commonly encountered with the hypervariable octameic oligonucleotide fingerprints (i.e., HOOF print) assay. **(A)** displays the GeneScan data for a three-plex primer combination. In the electropherogram display, the peaks corresponding to the internal size markers (ROX-label) have been subtracted. *(continued)*

The presence of substantial stutter peaks can obscure alleles in a multiplexed mixture. The allele for Locus-8 (shown in **Fig. 7A**, marked with a "Y" for yellow) can easily be overlooked among the stutter peaks from Locus-7. When the peaks from Locus-7 are subtracted from the electropherogram, the Locus-8 allele becomes prominent (*see* **Fig. 7C**). To a certain extent, color-coding of the peaks helps in sorting through the peaks. However, if the allele from one locus overlaps with stutter from another locus, the data could be difficult to interpret.

3.8.4. Overlapping Alleles

One of the problems associated with multiplex analyses is the potential for alleles to overlap, if both alleles are the same size. To minimize potential problems with interpreting data containing overlapping alleles, the primer cocktails were selected to contain primer pairs in different combinations so that if one combination overlaps, there is a good chance that the other combination will not overlap.

3.8.5. Bleed-Through Peaks and Spikes

Amplicon colors are determined by differences in the peak emission wavelengths of the four fluorescent dyes. 6-FAM emits a blue wavelength, HEX emits a green wavelength, NED emits a yellow wavelength, and ROX emits a red wavelength. During electrophoretic separation, the migrating DNA molecules are illuminated by an Argon laser as they pass by the detector, where they are scanned by four independent sensors, each set to detect light emissions of a specific wavelength matching the optimal wavelength of one of the dyes. Bleed-through peaks, also known as "pull-up" peaks, occur when the signal from one dye (e.g., blue) is picked up by the sensor for a different dye (e.g., green) so that the signal is read by both sensors as independent peaks.

Fig. 7. *(Continued)* The straight arrows point to the 6-carboxyfluorescein (FAM)-labeled peaks (blue). The largest peak is the true allele; the other FAM-labeled peaks are stutter peaks. Note that the peak heights of the stutter peaks (*y*-axis) are unusually large (>1000 relative fluorescent units) probably as the result of suboptimal conditions during amplification. **(B)** is an enlargement of the electropherogram in **(A)** after all the peaks, except the FAM-labeled peaks, have been subtracted. The symmetry of the products is a typical feature of stutter. **(C)** is also an enlargement of **(A)**; the FAM-labeled peaks have been subtracted, revealing a bleed-through peak from the FAM allele that appears as a false HEX-labeled peak. The false bleed-through peak also is recorded in the data table. A small HEX-labeled stutter peak also can be seen in **(C)**. These data also show the presence of Plus-A alleles. G, HEX (green) allele; Y, NED (yellow) allele.

Bleed-throughs can occur because of emission spectra of the four dyes overlap so that although most of the light is emitted at the optimal wavelength, small amounts of light are emitted over a wider range of wavelengths. Usually, this is not a problem because the amount of light emitted at wavelengths matching the other dyes is too small to be detected. But if a sample is overloaded, the amount of light emitted at nonoptimal wavelengths may be significant, triggering the sensor to register a nonexisting peak (see the "G" peaks in **Fig. 7A,C**). A clue that bleed-through may be occurring is the presence of a very large peak of one dye color superimposed directly over a smaller peak of a different dye color. Sometimes, these false signals will register with multiple sensors so that multiple dye peaks are superimposed. Bleed-through usually can be resolved by investigating inconsistent results for a given locus when it is multiplexed with different primer combinations. Another clue is that bleed-through peaks may not conform to the expected size of any allele for that locus.

"Spikes" are very sharp thin false peaks, often "multicolored," that may be confused with data peaks. They occur from a number of causes, including micro-air bubbles, dust, urea crystals or dried polymer flakes passing through the capillary, or from minor voltage fluctuations within the instrument. These artifacts may be noticed if the peak in question does not match an expected allele size or if it is found in only one of the duplicate tests for a primer pair. Because spikes are not reproducible in a sample, these artifacts can be identified by rerunning the sample.

3.8.6. Null Alleles and Microvariants

Sometimes spontaneous mutations will occur at a VNTR locus. These mutations produce alleles of unexpected sizes that are called microvariant alleles. These alleles can be produced by mutations that occur within the conserved DNA that flanks the tandem repeat regions or by irregular mutations that occur within the repeat areas. If the mutation occurs at a primer annealing site, it can prevent amplification of the locus. These are called "null alleles" because no amplicons are produced. Locus-4 is an example of a microvariant locus because an 11-bp insertion/deletion in the flanking DNA causes the alleles of *B. abortus* and *B. melitensis* to be different from the alleles of the other *Brucella* species ***(19)***. Microvariations have also been observed within Locus-1 and Locus-6.

Microvariants can make the genotyping data difficult to interpret. If an insertion/deletion happens to be a multiple of 8-bp, the locus will be assigned the wrong allele because the mutant product will mimic another allele in size. At the same time, some microvariants can be stable markers that help to identify genetic linkages among strains. For example, an unanticipated allele size at Locus-4 might indicate that the strain is a different species of *Brucella* than expected.

3.8.7. Inaccurate Size Calling

It is important to choose an internal size standard that covers the entire range of expected allele sizes, since size calling is only accurate for mobilities falling within the calibration curve. A sample calibration curve is shown (*see* **Fig. 8**). The relationship between size and mobility is not linear at the ends of the curve. Therefore, mobilities outside of or near the ends of the calibration curve are not called accurately. There is a gap of data points between approx 200 and 300 bp. This is because the 250-bp fragment runs anomalously in the POP4 separation matrix and is usually omitted from the standard curve calibration ***(23)***. However, without the 250-bp marker, the calibration curve is linear and should be able to adequately determine the size of fragments in this region.

Other causes of migration inconsistencies that can lead to inaccurate size calling involve small differences in the assay environment or machine performance such as run temperature, electrolyte concentration of the sample, and electric field strength ***(24)*** as well as the age and condition of the capillaries. Mobility also can be affected by the specific sequence, probably because of the formation of secondary structure, even in denaturing conditions ***(25)***.

3.8.8. Degraded DNA

Degradation of the DNA sample can negatively affect the success of the assay. Some degradation can be tolerated because the amplified products are relatively small. However, significant degradation of the target genomic DNA can cause a PCR amplification bias toward the smallest alleles, whereas larger alleles may not be amplified at all. One clue that indicates potential sample degradation is a noticeable decrease in peak height with increasing allele size at all loci. To prevent degradation of the DNA, samples should be stored at 4°C with a pH of 8.0 to 8.5. If the samples will be stored for the long term, or if it will be more than a few days before the cultured bacteria can be tested, it is best to keep the samples in a preservative that stabilizes DNA (*see* **Note 3).**

3.8.9. Identifying Microevolution vs Superinfection

During the development of the HOOF-print assay, we sometimes found that the capillary electrophoresis data for a particular strain consistently showed multiple peaks/amplicons at one or more loci. Further investigation showed that this resulted from mixed alleles in the sample and was not due to stutter or any other assay-related artifact. There are two categories of mixed alleles detected by this assay. One category is the variant alleles produced by microevolution. The accelerated mutation rates of VNTR loci cause some loci to continuously mutate (adding or losing repeat units). Unless the mutation event is harmful, the strain evolves into a cumulative population of genetically related bacteria with

GeneScan 3.7 - [F05_lys-5_101203.fsa]
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Best Fit 2nd Order Curve
A0 = -1.299521E+002
A1 = +6.891402E-002
A2 = +1.779974E-006
R^2 = 1.000
Size Calling Curve
Local Southern Method
Data Point
540
480
420
360
300
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180
120
60
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2100
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3500
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small genotypic differences. Characteristic of microevolution is that the population consists of one predominant genotype with a collection of variant genotypes that differ from the major genotype by a single (or small number of) repeat unit(s). Microevolution can be found within a single infected herd and even an individual animal. The longer the herd is infected, the more microevolution will progress in the bacterial population.

Mixed alleles also can arise from a completely different mechanism, whereby the disease is caused by a mixture of two unrelated strains of *Brucella*. Typically this is recognized by a combination of two very different genotypes in the herd or animal, which at some point in time arose from superinfection with two field strains. In some instances, the mixed strains are different species of *Brucella*. Occasionally, animals are co-infected with a vaccine strain as well as a field strain. These types of mixed infections are most commonly associated with enzootic regions where the disease is prevalent, widespread, and multiple strains persist.

The only way to determine the complete genotypic composition of a strain is to culture the *Brucella* and test multiple colonies, individually. The more colonies that are examined (ideally 10–20), the more information about strain variants and their frequencies can be determined. Testing the bacteria from multiple positive animals also helps to characterize an outbreak strain.

4. Notes

1. Separate sets of reusable pipettors must be used for pre-PCR setup and for downstream processes that involve the DNA targets or amplicons to prevent potential cross-contamination of samples or reagents.
2. The use of multichannel and repeating pipettors is not required for this protocol, but they are recommended because they can significantly decrease the amount of time needed to perform the procedure because many pipetting steps are involved and because they decrease the risk of misloading samples into the wrong wells in plates or gels.
3. Preserving samples in methanol is recommended for samples that will not be tested within 7 to 10 d. When preserved as described, samples can be stored at 4°C for at least 5 yr without noticeable deterioration. As an additional benefit, the bacteria are killed during preservation, reducing the risk of accidental infection of laboratory personnel and eliminating further need for BL3 containment.
4. These plates are sold as fully skirted, half-skirted, and nonskirted, referring to the size of the vertical edge surrounding the outside of the plate. The choice of skirt

Fig. 8. *(Opposite page)* Example of a calibration curve calculated by the local southern method from the mobilities of the internal size markers. For accurate sizing, the experimental amplicon sizes must fall within the linear range of the calibration curve. Because of the abnormal migration of the ROX-250-bp marker, this marker typically is not included in the calibration.

depends on the brand of thermal cycler and the brand of capillary electrophoresis system used since some brands do not provide enough clearance for all sizes of skirts.

5. When preparing Tris-derived buffers containing EDTA, the Tris component should be dissolved first and brought to a pH of 8.0 before adding the EDTA salt because EDTA is more soluble at a pH value of 8.0 or greater. EDTA is an acid; consequently, the pH value of the solution will need to be checked while the EDTA dissolves and maintained at 8.0. The inclusion of EDTA in certain buffers prevents DNA degradation by chelating Mg^{2+} ions that are essential cofactors for many enzymes, including nucleases. Mg^{2+} ions are also essential for DNA polymerase activity including Taq and other polymerases used for PCR. Therefore, it is important that dilutions of primers and target DNAs are made in water and not TE, since even small changes in the $MgCl_2$ concentration will affect the quality of the amplification reaction.
6. The choice of DNA polymerase is critical to the success of the assay because not all polymerases behave the same. First, the polymerase system chosen should have, or be compatible with, "hot start" capability. That is, the polymerase should be inactivated until the reaction mix is heated to at least 50°C. Second, the polymerase must amplify all targets equally well. A large number of polymerases were tested during the development of the assay with mixed results. Using the parameters specified in this chapter, the best-performing DNA polymerase was FastStart Taq DNA Polymerase by Roche Applied Science; however, other products may be equally suitable.
7. The forward primer for each VNTR locus is labeled with a fluorescent dye at the 5' end. The three dyes (HEX, NED, and FAM) were chosen to be compatible with fluorescent detection by the ABI Prism 3100 Genetic Analyzer with filter set D. If a different brand of fragment analyzer is chosen then the dye labels may need to be changed to meet the requirements of that analyzer.
8. Synthetic oligonucleotide primers should be resuspended for storage in TE buffer at a concentration of 200 pmoles/µL (*see* **Table 1**). The primers labeled with a fluorescent dye must be stored in the dark and protected from light as much as possible. Dispensing the primers into 50-µL aliquots will minimize loss if the stock becomes contaminated or mishandled. Synthetic primers, including the fluorescently labeled primers, are stable at 4°C for at least 3 yr if stored in TE and protected from light.
9. Fluorescent-tagged oligonucleotide primers are significantly more expensive than conventional primers. However, the cost is not proportional on the quantity of oligonucleotide synthesized. Therefore, it is only about twice as expensive to order an 80,000 pmol synthesis as it is to order a 10,000 pmol synthesis. Because the primers are stable for at least 3 yr if stored properly, it is more cost efficient to buy large quantities if possible.
10. Plastic cap strips are the most reliable method for sealing the wells of PCR plates, when the thermalcycler does not have a compression style hot bonnet. However, they can be difficult to align and properly seat into the wells. Exam-

ine the plate carefully to be sure that all of the caps are seated correctly and completely. It is recommended that the cap-strips be purchased from the same manufacturer as the PCR plates because not all brands of caps will fit into all brands of plates.

11. Be certain that the cap mat or sheet used to seal the plate is designed for PCR and also is designed for the style of plate being used. Some types of plastic film sheets do not seal well on plates with raised edges around the wells. Others cannot withstand the build up of air pressure when the sample is heated, and may partially detach from the wells allowing evaporation of the sample. We recommend using silicone cap mats (DOT Scientific) because they mold to the shape of any well style forming an inverted dome. However, it is good practice to seal the mat with a plate roller immediately before lowering the thermalcycler's bonnet because the silicone beads sometimes slip out of the wells when not under compression.
12. Many options for gel electrophoresis systems are available. We recommend the use of a bufferless system (e.g., E-Gel *96* mother or daughter base Invitrogen Corp.) with precast, prestained gels in plastic cassettes (e.g., E-Gel *48* gels with 4% agarose) because they perform very reproducibly, the plastic cassettes are easy to load with a multichannel pipettor, and no loading buffer with dye (that can potentially obscure the visibility of bands) is needed for the samples. These systems reduce exposure to hazardous chemicals and also reduce the amount of hazardous waste. However, any standard agarose gel electrophoresis system can be used. When preparing agarose gels, use only DNA grade agarose.
13. SYBR Gold Nucleic Acid Gel Stain is a dye with up to 10 times greater sensitivity than ethidium bromide. However, it cannot be used as a pre-electrophoresis stain because it alters DNA migration and it is absorbed onto glass and certain plastic surfaces. Carefully follow the manufacturer's instructions.
14. Ethidium bromide and SYBR Gold are light sensitive and should be protected from light. Either staining solution (at 1X concentration) can be stored for up to two weeks if stored in a light-proof container (e.g., covered with aluminum foil). Ethidium bromide can be stored at room temperature, whereas SYBR Gold should be stored at 4°C.
15. The quality of the formamide is critical for proper injection and running of the sample through the capillaries. Use only high purity (>99.5%) and low conductivity (<100 μmhos) reagent that has been deionized and stored at an alkaline pH.
16. Most *Brucella* species are Class III pathogens and may fall under special governmental rules and policies. In the United States, *Brucella abortus*, *B. melitensis*, and *B. suis* are listed as Veterinary Services Overlap Select Agents and therefore subject to the Code of Federal Regulations, Title 9, Parts 121 and 122. Regardless of the applicable governmental regulations, all steps involving the use of live *Brucella* should be done in an approved Biological Safety Cabinet with appropriate precautions.
17. Better results are obtained if samples containing methanol preserved bacteria are diluted with water and stored at 4°C overnight. However, once it has been diluted

in water, the DNA inside the bacteria is less stable and so the diluted samples should not be stored for longer than approx 1 wk.

18. Two loci, Locus-6 and Locus-8, are only tested once per sample. So far, these loci have shown very little polymorphism for *B. abortus* strains. For other *Brucella* species, these loci should also be tested in duplicate.
19. The lyophilized primer should be dissolved in TE buffer as a 1000X solution. Depending on the information reported in the certificate of analysis provided with custom synthesized primers, 1) add TE to the primer at the ratio given in **Table 1 column 3**, if the certificate reports the total number of micrograms synthesized or 2) add TE at a ratio of 200 pmol/µL if the certificate reports the total number of picomoles synthesized.
20. The 100-µL primer cocktail working solution is sufficient for up to three 96-well PCR plates (30 test samples).
21. VNTR Loci-1 to -8 have a conserved flanking sequence on one side of the repeat sequence, therefore the reverse primers Rev-1 and Rev-3 can be used with multiple forward primers.
22. Thorough mixing of the master mix is critical to the success of the assay because several components are very dense and will settle to the bottom by gravity if not properly dispersed.
23. It is helpful to use a strip of lab tape or masking tape to cover the wells on either side of the well-column to be filled. The tape serves the dual purpose of maintaining orientation and preventing potential contamination of adjacent wells with microdroplets of sample.
24. This protocol uses a higher than normal extension temperature based on empirical optimization of the assay conditions. It is possible that the higher temperature relaxes secondary structure formation in the target DNA, allowing the polymerase to extend through the potentially folded regions.
25. The first incubation step

is for activating DNA polymerases that have been chemically modified for hot start capability. The temperature and time should be adjusted for the specific DNA polymerase system used as recommended by the manufacturer. Many DNA polymerases do not need the precycle heating step for activation.

26. Incubating the amplicons for an hour increases the proportion of molecules bearing the nontemplated nucleotide addition by Taq and certain other DNA polymerases and consequently simplifies the fragment separation profile by minimizing one of the peaks in the doublet.
27. Place the agarose gel on the deck of the apparatus before loading the samples. Add 1X running buffer to approx 3 to 5 mm above the gel surface. Load the samples in a high density loading buffer at 1X concentration. If using a multichannel pipettor, be particularly careful not to pierce the gel or the bottom of the wells with the tips, as this can cause the sample to leak out of the well.
28. This program is designed to pick-up 22 µL but only dispense 20 µL to avoid producing bubbles in the wells. However, because the water is picked up first, very little sample is lost in the remaining 2 µL.

29. With an expandable pipettor, it is possible to use the wide channel spacing (9.0 mm) to remove the samples from a row of the 96-well plate, then collapse the channel spacing to an appropriate distance (e.g., 4.5 mm for E-gels) for dispensing the samples into the gel wells.
30. Gels enclosed in a plastic cassette are especially easy to load with a multichannel pipettor because the top plastic plate protects the surface of the gel from accidental puncture while aligning the tips with the wells. Some caution must be exercised to avoid puncturing the agarose at the bottom of the gel wells as this can lead to sample leakage between the gel and the lower plastic plate. Gently pressing the tips against one side of the wells will help stabilize the pipettor while dispensing the samples and assure that little residual sample will remain attached to the tips.
31. With more sizes and regular intervals, the ladder becomes more accurate. However, the creation of these ladders is very time consuming because many alleles must be amplified for each locus, the products must be quantified and the components assembled in appropriate ratios.
32. Data from small peaks (<100 RFU) should be repeated with more sample (a lower dilution of the PCR or, if the amplification was not sufficiently productive, with a new amplification reaction with more template).

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13

Detection of *Legionella* in Various Sample Types Using Whole-Cell Fluorescent *In Situ* Hybridization

Priscilla Declerck and Frans Ollevier

Summary

The human pathogenic *Legionella* bacteria are found ubiquitously in natural and human-made aquatic environments as residents in biofilms, where close interactions with other microorganisms like protozoa are possible. Nosocomial legionellosis already has been linked frequently to *Legionella*-contaminated artificial water supplies. For this reason, a rapid and accurate detection and quantification of these bacteria in environmental and clinical samples, combined with more information about their behavior in complex microbial communities and diverse ecosystems, is of importance. More insight into the ecology of the *Legionella* bacteria can lead to new methods to suppress their high numbers in human-made aquatic systems. Fluorescent *in situ* hybridization (FISH), based on ribosomal ribonucleic acid-targeted oligonucleotide probes, combines the precision and specificity of a molecular technique with the power to visualize individual cells without prior cultivation. In this chapter, the use of FISH for the detection and quantification of *Legionella* in water samples and in the visualization of these bacteria inside protozoa and biofilms is described in detail.

Key Words: ISH technique; 16S rRNA; probes; detection; quantification; *Legionella pneumophila*; protozoa; biofilms; epi-fluorescence microscopy; CLSM; FITC; Cy3.

1. Introduction

The genus *Legionella* belongs to the *Legionellaceae* and comprises more than 42 species ***(1,2)***. The bacteria are ubiquitous in various natural and artificial aquatic environments, where they reside in complex microbial communities called biofilms and where they replicate intracellular in certain protozoa ***(3)***.

The diseases caused by *Legionella* are collectively termed legionellosis and comprise the self-limiting Pontiac Fever and the potential deadly Legionnaires' disease. *L. pneumophila* is the etiological agent of more than 90% of the legion-

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ellosis infections ***(1)***. Infection happens when aerosols (<5 μm) containing free-living bacteria escape from a used protozoan host or a biofilm, or excreted *Legionella*-filled vesicles derived from protozoa or protozoa harboring bacteria are inhaled ***(4)***. People most at risk for infection are elderly persons and immunocompromised patients. Because of the high percentage of people in these categories, the number of individuals susceptible to infection has increased.

Several studies already showed a clear association between the presence of *Legionella* in human-made aquatic systems and the occurrence of legionellosis outbreaks ***(5)***. Therefore, it is of importance not only to be able to detect and to quantify these bacteria in environmental and clinical samples but also to gain a better insight in their ecology. More information about their *in situ* activity and temporal distribution inside protozoa and biofilms can lead to methods to control the high *Legionella* numbers in human-made aquatic systems. These days, molecular techniques such as polymerase chain reaction (PCR) and fluorescent *in situ* hybridization (FISH) are popular diagnostic tools. In contrast to the PCR technique, FISH combines the precision of molecular genetics with the visual information from microscopy to permit the visualization and identification of individual microbial cells within their natural microhabitat ***(6)***. The FISH technique includes several general steps, namely, fixation of the sample, further preparation of the sample before the hybridization, hybridization using specific ribosomal ribonucleic acid (rRNA) probes, removal of unbound probes, mounting, visualization, and analysis of stained samples ***(6)***. Because FISH is fast and easy to conduct, it frequently is used in the *Legionella* research in our laboratory ***(7,8)***.

In this chapter, the application of FISH in the rapid and accurate detection of *Legionella* in water samples and in the visualization of their presence in biofilms and in protozoa hosts is described in detail. To visualize the *Legionella* bacteria in the various kinds of samples, specific rRNA-targeted and -labeled gene probes that bind to the 16S rRNA target of *Legionella* and *L. pneumophila* are used. Because of the high copy number of 16S rRNA in each replicating and metabolically active cell, the 16S rRNA offers the greatest ease and accuracy in identification for most microorganisms ***(6)***. Because the oligonucleotide probes are labeled with different fluorescent dyes, it is possible to simultaneously detect *Legionella* and *L. pneumophila* in the same sample. After staining the bacteria, samples are analyzed by means of epi-fluorescence or confocal laser scanning microscopy.

2. Materials

2.1. Laboratory Equipment

1. Class II safety cabinet for all manipulations with *Legionella*.
2. Bunsen burner.
3. 46°C incubator.

4. Isotonically equilibrated humid chambers.
5. Microlitre pipets (1–1000 µL).
6. Timer.
7. Hood.

2.2. Reagents and Buffers

1. Commercially labeled specific *Legionella* and *L. pneumophila* oligonucleotide probes (Eurogentec, Belgium), preserved at –20°C in TE buffer (10 m*M* Tris, 1 m*M* ethylene diamine tetraacetic acid, pH 7.5; *see* **Table 1** ***[9,10]***). These probes are labeled with a cyanine dye, namely Cy3, or with fluorescein-isothiocyanate (FITC; *see* **Notes 1** and **2**). Probe working solutions: 0.01 µg of Cy3-labeled probe per microliter of double-distilled water and 1 µg of FITC-labeled probe per microliter of double-distilled water (*see* **Note 3**).
2. Mineral oil type DF (Cargille Laboratories; *see* **Note 4**).
3. Sterile 1X phosphate-buffered saline (PBS): 40.5 mL of 0.2 *M* Na_2HPO_4, 9.5 mL of 0.2 *M* NaH_2PO_4, pH 7.4.
4. Paraformaldehyde fixation buffer (PFA): 2 g of paraformaldehyde, 32.5 mL of sterile double-distilled water, 1 *M* of sterile NaOH, and 16.5 mL of PBS, pH 7.2 (*see* **Note 5**).
5. Hybridization buffer: 360 µL of 5 *M* NaCl, 40 µL of 1 *M* Tris-HCl, pH 8.0, 400 µL of formamide, 1200 µL of double-distilled water, 2 µL of 10 % (w/v) sodium dodecyl sulfate (*see* **Note 6**).
6. Washing buffer: 360 µL of 5 *M* NaCl, 40 µL of 1 *M* Tris-HCl, pH 8.0, 1200 µL of double-distilled water, 2 µL of 10 % (w/v) sodium dodecyl sulfate.
7. 50, 80, 96, and 100% (v/v) ethanol (EtOH).
8. Double-distilled water.
9. Antifading product: Citifluor AF2 (Citifluor Ltd.,UK).

2.3. In Situ *Detection and Visualization of the Spatial and Temporal Arrangement of* Legionella *in Protozoa*

1. Microscope slides with eight reaction fields (Immuno-Cell Int., Mechelen, Belgium).
2. Confocal laser scanning microscope (CLSM; IX70, Olympus).
3. Argon-ion laser (163-C1210, Spectra Physics).
4. HeNe laser (05-LGP-193, Melles Griot) is used.
5. 50X and 100X Oil immersion UPlanApo objectives are necessary for the detailed scanning of the samples.
6. Fluoview FV500® program (Olympus, Belgium).

2.4. Detection and Quantification of Legionella *in Water Samples*

1. A filtration system (Millipore).
2. A vacuum pump (Millipore).
3. Isopore™ membrane polycarbonate filters (0.2 µm, Ø: 47 mm, GTTP) (Millipore).
4. Sterile funnels (poly-ethylene, 100 mL; Millipore).

Table 1
Details of Oligonucleotide Probes Used in the Fluorescence *In Situ* Hybridization Analysis of *Legionella* Samples

Probe	Target	Sequence (5′ → 3′)	Target site	Formamide %	Reference
LEGPNE1	*L. pneumophila*	ATC TGA CCG TCC CAG GTT	16S	20	***9***
LEG705	Most *Legionellaceae*	CTG GTG TTC CTT CCG ATC	16S 705–722	20	***10***

5. Plastic jars.
6. Tweezers.
7. Scalpel.
8. Microscopic slides (Knittel Gläser, Germany).
9. Cover slips (24 × 60 mm) (Mensel Gläser, Germany).
10. Epifluorescence microscope (BX 51, Olympus) equipped with a 100-watt mercury lamp.
11. 50X and 100X Oil immersion UPlanApo objectives used in combination with fluorescence filter sets U-M41007 (HQ-Cy3: 535-565) and U-MWIBA_2 (460–490) (*see* **Note 7**).
12. Fluorescence camera (DP 50, 6×10^6 pixels)
13. DP-soft program (Olympus, Belgium).

3. Methods

3.1. In Situ *Detection and Visualization of the Spatial and Temporal Arrangement of* Legionella *in Protozoa*

The FISH staining can be used in infection assays of *Legionella* and protozoa ***(8,11)***.

1. Clean a microscopic slide with reaction fields using 100% EtOH.
2. Centrifuge 1 mL of each cell suspension containing the infected protozoa for 10 min at 128*g*.
3. Discard the supernatant carefully and resuspend the pellet in 200 µL 1X PBS.
4. Apply 20 µL of cell suspensions in each well of the cleaned microscopic slides.
5. Air dry (*see* **Note 8**).
6. Add 20 µL of PFA buffer (*see* **Note 9**).
7. Fix for 1 h at room temperature in an isotonically equilibrated humid chamber.
8. Remove buffer and wash with PBS.
9. Dehydrate the slides in an aqueous series of 20 µL 50, 80, and 96% EtOH for 3 min each and air dry (*see* **Note 10**).
10. Prepare for each desired probe a probe solution consisting of (*see* **Note 11**): 30 ng of Cy3-labeled probe/20 µL hybridization buffer; and 100 ng of FITC-labeled probe/20 µL hybridization buffer.
11. Add 20 µL of the desired probe solution onto each well of the dehydrated slides (*see* **Note 12**).
12. Incubate the slides for 1.5 h at 46°C in an isotonically equilibrated humid chamber (*see* **Note 13**).
13. Place washing buffer at 46°C.
14. Remove hybridization buffer gently.
15. Wash slides twice with 20 µL of the preheated washing buffer (*see* **Note 14**).
16. Apply 20 µL of washing buffer and incubate the slides for 20 min at 46°C in a humid chamber.
17. Rinse carefully with double distilled water and air dry in the dark.
18. Add a few drops of Citifluor before microscopic analysis or store the slides at –20°C without Citifluor (*see* **Note 15**).

19. Cover with 24 × 60-mm cover slips and press the cover slip gently to the slide, so that the Citifluor covers each well.
20. Analyze the slides using a CLSM using mineral oil when working with the 50X and 100X objectives.
21. Slides can be analyzed more than once by gently removing the cover slip, rinsing most of the Citifluor with double distilled water, air dry and store the slides at –20°C.
22. Before re-analysis of the slides from –20°C, let them first re-adapt to room temperature and if necessary add a few drops of Citifluor.

3.2. In Situ *Detection and Visualization of the Spatial and Temporal Arrangement of* Legionella *in Biofilms*

This method can be used for the detection of *L. pneumophila* in biofilms grown on any kind of slide material (*see* **Note 16**).

1. Rinse the slides, with the attached biofilms, three times in PBS to remove unattached microbial cells.
2. Place each slide immediately in PFA buffer.
3. Fix for at least 2 h (*see* **Note 17**).
4. Perform the hybridization as described for the *in situ* detection of *Legionella* in protozoa (*see* **Subheading 3.1.**), adjusting the amount of probe solution of the slide surface.

3.3. Detection and Quantification of Legionella *in Water Samples*

1. Sterilize the filtration surface of each unit of the filtration system with 100% EtOH.
2. Place a filter membrane (with the shiny side on top) on each filter unit.
3. Put on a sterile funnel.
4. Poor 100 mL of each *Legionella*-contaminated water sample into a funnel, turn on the filtration system at –5 kPa and refill the funnels if necessary.
5. After filtration, turn off the pump and close each filter unit, overlay each membrane with 10 mL of 4% PFA buffer for 1 h.
6. Discharge the PFA buffer by turning the pump on.
7. Wash the filters twice with 1X PBS.
8. Dehydrate through a 50 to 96% EtOH series.
9. Remove the funnels and let each filter air dry.
10. Before staining, cut the filter in four pieces by using the scalpel.
11. Bring each piece on a precleaned microscopic slide.
12. Apply 20 µL of the probe solution and hybridize (*see* **Subheading 3.1.**).
13. Remove the hybridization buffer by putting the filter pieces on tissue and wash with washing buffer.
14. Put the pieces into plastic jars filled with washing buffer and incubate for 20 min at 46°C.
15. Remove the washing buffer by putting the filter pieces on tissue and rinse them one last time with double distilled water.

16. Let the filters dry thoroughly and analyze them or store them at –20°C.
17. For the microscopic analyses put each filter piece on a cleaned slide (100% EtOH) with a few drops of Citifluor.
18. For the quantification of the cells, scan each filter piece and count each field using an objective with a counting grid (*see* **Note 18**).

4. Notes

1. In the current protocol, the Cy3 and FITC staining are described. However, there are many other possibilities to use other direct labels (e.g., like Cy5, TexasRed; consult http://www.probes.com/handbook/). The choice of the labels frequently depends on the available filter sets of the fluorescence microscope.
2. In environmental samples, the detection of cells is achieved more easily with Cy3 or Cy5 fluorochromes because these labels are much brighter and more stable than the classic fluorescein and rhodamine-derivates.
3. Kept at –20°C, the probe stock solutions, like the working solutions, are stable for years. It is recommended that one divide the working solutions into small aliquots to prevent frequently thawing and freezing of the oligonucleotide probes.
4. When analyzing fluorescent samples, it is important to choose immersion oil, which exhibits a very low or virtually zero background fluorescence.
5. Always prepare this solution under the hood. Once divided into aliquots and frozen at –20°C, the PFA solution is stable for up to 1 yr. When thawed, the PFA has to be stored at 4°C. At this temperature, the PFA is stable for a few weeks.
6. Hybridization buffer and washing buffer need to be freshly prepared every time.
7. Fluorescence filters are always a compromise between selectivity and throughput. Single-band filter sets provide the best compromise between these two criteria, whereas multiple-band filter combinations can be used for the simultaneous observation of several dyes.
8. It is recommended that one always air dry the slides in a flow to prevent the sticking of dust particles onto the slides. This sticking can disturb the microscopical analysis because of the possible auto-fluorescence of some of those particles.
9. Gram-negative bacteria like *Legionella* are normally fixed in PFA buffer and need no additorial permeabilization before hybridization. Gram-positive cells can be fixed in 50% EtOH and permeabilized by exposure to lysozyme ***(12)***.
10. At this point, the dehydrated slides can be hybridized immediately or stored free of dust at room temperature for up to 3 wk until staining. If longer storage is needed, slides are stable at –20°C for several months.
11. From this step forward, avoid light as much as possible.
12. For double staining using both the LEG705 Cy3 and the LEGPNE1 FITC probes, add 10 µL of each probe solution, mix gently in the well and proceed the hybridization as described for single staining.
13. The hybridization of the samples must always be performed in a moist chamber to minimize the evaporation of water, which would otherwise alter the hybridization conditions in an uncontrolled manner.

14. During the washing steps, it is important to prevent slide surfaces from drying out; otherwise, background problems may arise.
15. One significant problem when using fluorescent dyes is bleaching of the fluorescence signal while being analyzed over time. Exposure times of minutes or even several seconds may have a critical effect on the signal destruction. This problem can be reduced by mounting the sample in an antifade solution such as Citifluor. Other suggestions to avoid the rapid destruction of the fluorescence signal are the use of narrow band filters and photostable dyes.
16. For the analysis of FISH stained *Legionella* in environmental samples, such as in biofilms or in filtered water samples, disturbing auto fluorescence signals can be present. These signals can originate from micro-organisms like moulds, yeasts, or bacteria such as *Pseudomonas* but also from the surrounding biofilm materials like biological and inorganic debris or algae. It is recommended that one use narrow-band filters and that one check first for auto fluorescence when dealing with environmental samples.
17. After the fixation step, biofilms can be stored at 4°C in PBS for a maximum of 3 d.
18. Although normally highly abundant, the rRNA content of bacterial cells may vary considerably, not only between species, but also between cells of one strain according to their physiological state, which is directly correlated with their growth rate ***(13)***. Low physiological activity can thereby result in low signal intensity or false-negative results. This problem can be solved by placing the filters on a growth medium that stimulates the rRNA production in the bacteria cells. Another solution is to use two specific probes that each target a different position of the 16S rRNA and that are labeled with different fluorochromes. However, this approach is restricted by the limited availability of specific target sequences for the respective micro-organism.

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14

Identification of Diagnostic Proteins in *Mycobacterium avium* subspecies *paratuberculosis* by a Whole Genome Analysis Approach

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Summary

Mycobacterium avium subspecies *paratuberculosis* (*M. paratuberculosis*) is an economically significant veterinary pathogen that causes Johne's disease in cattle and sheep. There is a critical need for improved diagnostic tests to detect *M. paratuberculosis* infection in these animals. As with many other animal diseases, efforts need to be concentrated on the development of simple, rapid, noninvasive tests that can be performed by veterinarians or animal producers without expensive laboratory equipment. With the genome sequence of *M. paratuberculosis* now complete, we have taken a different strategy to identify novel proteins that are present uniquely in *M. paratuberculosis* and are antigenic in the context of infected cattle. Through a whole genome comparison of *M. paratuberculosis* with other sequenced mycobacterial genomes, we identified a collection of more than 90 genes that are present uniquely in *M. paratuberculosis*. This list has been further trimmed to 39 after amplification using polymerase chain reaction of unique genes using the genomic deoxyribonucleic acid template from several mycobacterial species and isolates. A selection of the remaining genes has been cloned and expressed in *Escherichia coli* and purified by affinity chromatography. Successfully purified proteins were analyzed using sera from rabbits immunized with *M. paratuberculosis*. Furthermore, to identify antigens in the context of disease, sera from cattle with Johne's disease as well as healthy control cattle are used in immunoassays. Using this methodology, we identified the first protein antigens specific to *M. paratuberculosis.*

Key Words: Johne's disease; genomics; *Mycobacterium avium* subsp. *paratuberculosis*; protein antigens; genome sequencing; molecular diagnostics.

1. Introduction

Diagnosis of Johne's disease, caused by *Mycobacterium avium* subspecies *paratuberculosis* (*M. paratuberculosis*), is difficult because of the extremely

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slow growth in culture medium (20-h generation time) and the lack of a specific assay that is sensitive enough to detect infected animals at early stages or at least before shedding of the bacterium in the feces. Contamination is an added problem as *M. paratuberculosis*, a significant veterinary pathogen of cattle and sheep, is cultured from fecal specimens. The very high sequence similarity between *M. paratuberculosis* and other mycobacteria has confounded the development of diagnostic tests that specifically identify *M. paratuberculosis*-infected animals. Immunological tests for diagnosis of Johne's disease such as enzyme-linked immunosorbent assay and interferon gamma production historically have used complex protein mixtures, including a whole-cell sonicated protein preparation, cell wall prep, or a purified secreted protein fraction. Unfortunately, these preparations not only contain crossreactive proteins from closely related mycobacteria, but their preparation can vary considerably among, and even within, laboratories. With the *M. paratuberculosis* K-10 genome recently sequenced and annotated ***(1)***, more than 90 predicted coding sequences already have been identified as unique to *M. paratuberculosis* when compared with the sequenced but unannotated *M. avium* subspecies *avium* (*M. avium*) genome (available from http://www.tigr.org/tdb/mdb/mdbinprogress.html). To address the current lack of sensitivity and specificity in diagnosis of Johne's disease, we used a comparative genomic approach to identify all *M. paratuberculosis* sequences that are absent in the genetically similar *M. avium* genome ***(1a)***. However, comparative genomics via *in silico* analysis is not enough to identify a diagnostic sequence. The specificity of deoxyribonucleic acid (DNA) sequences identified by this approach must then be further tested by amplification via polymerase chain reaction (PCR), Southern hybridization, and whole genome array analysis with other species of mycobacteria, including *M. tuberculosis*, *M. bovis*, *M. leprae*, *M. gordonae*, *M. africanum*, *M. fortuitum*, *M. silvaticum*, *M. intracellularae*, and other known mycobacterial species ***(2)***. Additional *M. avium* and *M. paratuberculosis* isolates also were used to confirm that sequences are present in all *M. paratuberculosis* and absent in all *M. avium* isolates. These analyses have yielded a complete catalog of coding sequences that are present only in *M. paratuberculosis*. This method is readily adaptable to other bacterial species with sequenced genomes.

The development of immunoassays specific for the diagnosis of Johne's disease requires antigens unique to *M. paratuberculosis*. Therefore, all coding regions present in *M. paratuberculosis*-specific DNA fragments are cloned and expressed in *Escherichia coli* or *Mycobacterium smegmatis*. The expressed recombinant protein is then affinity purified from bacterial lysates. As described herein, the system that has worked best in our hands is the maltose binding protein (MBP) system in *E. coli*, but other expression systems should also work well. Finally, expressed proteins can be evaluated with a panel of sera from

naturally infected and control animals to determine which recombinant proteins are the best antigens in the context of infection.

The rapid development of mycobacterial genomics after the completion of eight genome sequences (*M. paratuberculosis*, *M. leprae*, *M. tuberculosis* H37Rv and CDC1551, *M. bovis* AF2122/97 and BCG-Pasteur, *M. avium*, and *M. smegmatis* ***[3–5]***) will provide the basis for powerful new approaches to identify species-specific sequences. By identifying common sequences as well as unique regions within each mycobacterial genome, a better understanding of the genetic requirement necessary to cause mycobacterial diseases also will emerge. Hence, advances made by a whole genome analysis of *M. paratuberculosis* will not only increase understanding of Johne's disease but also will contribute significantly to comparative mycobacterial genomics in general. Finally, the identification of genes that are specific for *M. paratuberculosis* and proteins expressed from these genes are likely to be excellent candidates for diagnostic tests and vaccine development.

2. Materials

2.1. Computer and Software

To perform the concatenated Basic Local Alignment Search Tool (BLAST) analyses ***(6)*** and other in silico comparative approaches a computer with minimum specifications of 256-Mb RAM and a Pentium 4 processor or equivalent is recommended. Using a computer system with additional RAM (1 Gb or greater) and/or multiple processors will significantly improve the speed of the analyses, especially when working with large or multiple complete genome sequences. Additionally, a minimum of 20 Gb hard drive space is recommended to accommodate the storage of local sequence databases and analysis results.

Artemis is a genome sequence and annotation viewer ***(7)***, and ACT is a DNA sequence comparison tool based on Artemis. Both programs are free and available for downloading from the Sanger Center (http://www.sanger.ac.uk/Software/Artemis/ and http://www.sanger.ac.uk/Software/ACT/). BLAST is freely available from the National Center for Biotechnology Information (ftp://ftp.ncbi.nlm.nih.gov/blast/). All of these programs are available for different types of operating systems.

2.2. PCR Analysis of Mycobacteria

1. Standard amplification reagents are used (Taq polymerase, oligonucleotide primers, Taq buffer, template DNA).
2. Dimethyl sulfixode (up to 5%) is added to aid in amplification of GC-rich sequences.
3. Restriction enzymes such as *Xba*1, *Hind*III, Ligase supplied with NEB2 buffer (New England Biolabs)

2.3. Protein Expression–Purification

1. Expression vector: pMAL-c2 (New England Biolabs).
2. *Escherichia coli* DH5-α or other suitable strain.
3. LB plates and medium containing 0.2% glucose and 100 µg/mL ampicillin.
4. 1 *M* isopropyl-1-thio-β-D-galactopyranoside (IPTG): aliquoted and stored at –20°C.
5. Elution buffer: 10 m*M* maltose, 20 m*M* Tris-HCl, pH 7.4, 0.2 *M* NaCl, 1 m*M* EDTA, 10 m*M* 2-mercaptoethanol.
6. Column buffer: 20 m*M* Tris-HCl, pH 7.4, 0.2 *M* NaCl, 1 m*M* EDTA, 10 m*M* 2-mercaptoethanol.
7. Coomassie brilliant blue stain or GelCode Blue (Pierce Immunochemical).
8. Amylose resin (New England Biolabs).
9. Sonicator (Tekmar sonic disruptor equipped with a microtip tissue disruptor).
10. Column to hold resin (2.5 × 10 cm).

2.4. Immunoblot Assay

1. Nitrocellulose filters.
2. Blot trays or Petri dishes to hold nitrocellulose filters during washes and exposure to antibodies.
3. Phosphate-buffered saline (PBS).
4. Blot wash solution: PBS, 0.1% Tween-20.
5. Blot block solution: PBS, 0.1% Tween-20, 2% bovine serum albumin. Store at 4°C.
6. Primary and secondary antibodies: secondary antibodies are conjugated to horseradish peroxidase.
7. SuperSignal detection reagent (Pierce.).
8. X-ray film (Kodak).
9. Saran wrap.
10. Bio-Rad transblotter or semidry blotter.
11. Casting plates and stands for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels (Bio-Rad).
12. Acrylamide solution (12% running gels and 4% stacking gels).
13. SDS-PAGE loading dye (1X): 1% SDS, 50 m*M* Tris-HCl, pH 6.8, 1% 2-mercaptoethanol, 10% glycerol.
14. SDS-PAGE running buffer (1X): 25 m*M* Tris-base, 250 m*M* glycine, 0.1% SDS pH 8.3.
15. Blot/transfer buffer: 25 m*M* sodium phosphate, pH 7.8.

3. Methods

3.1. Bioinformatic Analysis of the *M. paratuberculosis* Genome to Identify Candidate Diagnostic Sequences

All unique *M. paratuberculosis* sequences can be identified using BLASTN and MEGABLAST searches, for comparison of the *M. paratuberculosis* K10 and *M. avium* genomes. The output of these searches can be visualized with

ARTEMIS and ACT software. Sequences present uniquely in *M. paratuberculosis* (as determined by having no matches between the two genomes with an E-value below an established cutoff) can then be used to query a local installation of the Genbank nonredundant protein database using TBLASTX. If no significant sequence alignments are observed after this analysis, the sequence is marked as a candidate diagnostic sequence.

3.2. Verification of Potentially Diagnostic Coding Sequences

Analyses of candidate sequences not present in *M. avium* can be expanded using PCR amplification of several mycobacterial species as well as closely related bacterial species to determine whether specificity is maintained throughout the genus and beyond. To accomplish this, primers are designed from all identified unique predicted coding sequences and used in amplification reactions with DNA from other mycobacterial species and other closely related genuses. A positive control amplification reaction should involve a highly conserved sequence such as 16 rRNA ***(8)***.

1. Into a 500-µL PCR reaction tube, set up the following reaction (*see* **Note 1**):

   5.0 µL of 10X PCR buffer
   1.0 µL of PCR nucleotides
   2.0 µL of dimethyl sulfoxide
   1.5 µL of primer no. 1
   1.5 µL of primer no. 2
   38 µL of distilled deionized H_2O
   0.5 µL of template (mycobacterial genomic DNA 0.2 mg/mL)
   0.5 µL of Taq
2. Perform the PCR using the following conditions. A denaturation step at 95°C for 4.5 min, then 30 cycles at 95°C for 0.5 min, 58°C for 1 min, and 72°C for 1.5 min. Finally, add a finishing step at 72°C for 7 min.
3. Analyze the reaction by loading sample on a 1% agarose gel containg 0.1 µg ethidium bromide and conduct electrophoresis at 90 V for 1 h.

3.3. Production of Heterologously Expressed Proteins From Diagnostic Coding Sequences

3.3.1. Bioinformatics Analysis

Once a complete catalog of *M. paratuberculosis*-specific sequences has been assembled, each should be tested using DNA sequence analysis software. The predicted open reading frames should be confirmed and examined for motifs, such as the presence of potential signal peptides or membrane spanning domains and other characteristics such as hydrophilicity, surface probability, and antigenic index (using a suite of computer programs expressly designed for these purposes, e.g., DNAStar, GCG, BLOCKS, MOTIF) to identify the most likely candidate genes that may encode *M. paratuberculosis* antigens.

3.3.2. Cloning-Specific Coding Sequences Into the Expression Vector

Coding sequences within *M. paratuberculosis*-specific DNA fragments are cloned into *E. coli* expression vectors. In our hands, the use of the MBP vectors has enabled a higher percentage of expressed *M. paratuberculosis* proteins relative to the his-tagged system (*see* **Note 2**). The MBP system allows the expression and purification of a mycobacterial gene of interest by fusing it to the *malE* gene of *E. coli*, which encodes the MBP affinity tag.

1. Design primers that amplify the entire coding sequence. The 5´ primer should be designed with a *Xba*I restriction site and the 3´ primer with a *Hind*III restriction site for directional cloning into the pMAL-c2 expression vector. The gene should be amplified using conditions described in **Subheading 3.2.**
2. Clean up the reaction using Gene Clean or gel-purify the PCR product (*see* **Note 3**).
3. Cut both the PCR product and pMAL-c2 vector with *Xba*I and *Hind*III by preparing restriction reactions as follows:

10 µL of pMAL-c2 (0.16 µg/µL)	10 µL of PCR product (diluted to 0.5 mg/mL)
3 µL of 10X NEB no.2	3 µL of 10X NEB no.2
2 µL of *Xba*I (40 U)	2 µL of *Xba*I (40 U)
2 µL of *Hind*III (40 U)	2 µL of *Hind*III (40 U)
0.5 µL of 100 X BSA	0.5 µL of 100X BSA
12.5 µL of dH_2O	12.5 µL of dH_2O
30 µL total	30 µL total

4. Incubate at 37°C for 1.5 h.
5. Load digestion onto a preparative agarose gel and Gene Clean each digestion.
6. Run another gel to check yield of gel purified digestions. Based on that gel, set up the ligation reaction bringing the final volume up to 10 µL with dH_2O as follows:

Experimental	**Control**
1 µL of cut pMAL-c2 (0.16 µg/µL)	1 µL of cut pMAL-c2 (0.16 µg/µL)
4 or 5 µL of cut PCR product	4 or 5 µL of dH_2O
2 µL of 5 X Ligase buffer	2 µL of 5X Ligase buffer
1 µL of T_4 DNA ligase	1 µL of T_4 DNA ligase

7. Ligate overnight at 16°C or for 5 min at room temperature.
8. Transform *E. coli* DH5α frozen competent cells with approx 4 µL of the control and experimental ligation reactions using chemically competent cells. Plate the transformed cells on LB supplemented with glucose (0.2%) and ampicillin (100 µg/mL).
9. The next day, set up a PCR-based screen for several of the ampicillin-resistant colonies on the experimental plates to test for the presence of the insert as outlined in the following steps.
10. Pick a colony with a toothpick and swirl in a GeneAmp 0.5-mL microcentrifuge tube containing 25 µL of H_2O. Then plate it to a fresh antibiotic containing plate and place in incubator. Repeat this nine times with a different colony and fresh GeneAmp tube.

11. Heat the tubes to 100°C for 1 min in a heating block.
12. Centrifuge the 10 tubes at 20,000*g* for 2 min.
13. Remove 15 µL of the supernatant to a new tube for PCR.
14. Prepare the PCR master mix as follows. Ideally, 10 colonies should be tested with 25 µL of final reaction volume:

11 colony screen
75 µL of dH_2O
3 µL of primer no.1
3 µL of primer no.2
30 µL of 10X Taq PCR buffer
3 µL of *Taq* polymerase
6 µL of PCR nucleotides
120 µL

15. Add 10 µL of the master mix containing appropriate primers to each of the miniprep tubes.
16. Add 1 drop of PCR oil (silicone oil or mineral oil) to each tube.
17. Perform PCR making sure to include a positive control.
18. Analyze amplification products on an agarose gel.
19. Colonies that are PCR positive for the insert are sequenced to be certain the construct is correct and the coding sequence is in-frame with *malE*.

3.3.3. Expression and Purification of Recombinant Proteins

E. coli harboring the plasmid construct are cultured in 1 L of LB media supplemented with glucose (0.2%) and ampicillin (100 µg/mL) and induced with 300 µL of 1 *M* IPTG once the cells reach an OD_{600nm} of 0.4 to 0.6. After a 2-h induction, cells are harvested by centrifugation (*see* **Note 4**). A lysate of the harvested cells can then be produced by freeze–thaw cycles and brief sonication. Heterologously expressed mycobacterial proteins are then affinity purified from *E. coli* lysates by using amylose resin. A detailed protocol for this method has already been published ***(9)***, and a modified version also is detailed here:

1. Inoculate 10 mL LB/glucose/ampicillin medium with a single colony containing the fusion plasmid. Grow overnight at 37°C with shaking.
2. Inoculate 1 L of LB/glucose/ampicillin medium with 10 mL of overnight culture (a 1:100 dilution). Grow at 37°C, with shaking, to OD_{600nm} = 0.4 to 0.6.
3. Add 300 µL of 1 *M* IPTG to the remainder of the culture. Incubate 2 h at 37°C with shaking.
4. Centrifuge the remaining cells 20 min at 4000*g*, 4°C, and discard the supernatant.
5. Resuspend the cells in 40 mL of column buffer and freeze overnight at –20°C.
6. Thaw cell suspension and place on ice. Sonicate for 3 × 1-min bursts at 30 watts with a 2-min incubation on ice between each burst.
7. Centrifuge the sonicated cells 20 min at 14,000*g* at 4°C, and discard the pellet.
8. Pour amylose resin in a 2.5 × 10-cm column and wash with 8 column volumes of column buffer (*see* **Note 5**).

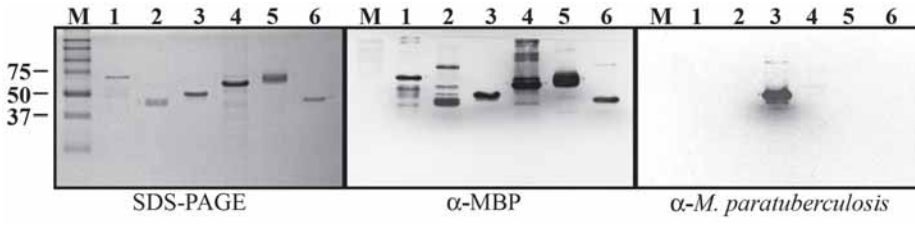

Fig. 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis of five *M. paratuberculosis* fusion proteins expressed in *E. coli*. The purified fusion proteins were separated by SDS-PAGE, electroblotted onto nitrocellulose, and probed with the antibody indicated beneath each immunoblot. α- maltose binding protein (MBP) is a monoclonal antibody (diluted 1:5,000) that binds to the MBP affinity tag. α-*M. paratuberculosis* is a rabbit antibody (diluted 1:1,000) against heat-killed whole paratuberculosis cells. Note that rabbit antibodies detected the purified fusion protein in lane 3 (MBP/MAP2762c). MBP alone, which is the fusion partner in these recombinant proteins, is present in lane 6. Lane assignments: M, protein size markers; 1, MBP/MAP2761c; 2, MBP/MAP2764c; 3, MBP/MAP2762c; 4, MBP/MAP2751; 5, MBP/MAP2753; 6, MBP alone.

9. Dilute sonicated extract 1:5 with column buffer. Load at a flow rate of approximately 1 mL/min.
10. Once all of the extract has been loaded onto the column, wash with 8 column volumes of column buffer.
11. Elute fusion protein with column buffer containing 10 m*M* maltose by collecting 12 0.5-mL fractions (*see* **Note 6**).

Collected fractions from the amylose column are analyzed by SDS-PAGE (*see* **Subheading 3.4.2.**) and spectrophotometrically at OD_{280nm}. The most concentrated samples are pooled and dialyzed (Pierce dialysis cassettes) in 1 L of PBS with three exchanges at 4°C. A selection of *M. paratuberculosis*-MBP fusion proteins purified using this method is shown in **Fig. 1**.

3.4. Analysis of Recombinant Proteins With Sera From Control and Infected Animals

Purified proteins produced in **Subheading 3.3.3.** can be evaluated serologically by immunoblot or enzyme-linked immunosorbent assay with a panel of sera from infected and control animals to determine whether the protein is detected by antibodies from infected hosts. The immunoblot method is described in **Subheading 3.4.3.** (*see* **Fig. 1**).

3.4.1. Preparation of Antigen/Protein Samples

1. Recover the purified MBP-fusion protein from dialysis. Remove a 20-µL aliquot and mix with an equal volume of 2X SDS–PAGE loading buffer so that the final concentration is 1X loading buffer.
2. Boil samples for 5 min. Samples are ready to be loaded onto SDS-PAGE gels or stored at –20°C. Freeze–thaw cycles are not a concern at this point.
3. With each new antigen sample prepared, empirically determine how much volume to load on individual SDS-PAGE gels. Sample concentrations can be roughly determined by staining gels with Pierce's GelCode Blue or Coomassie stain.

3.4.2. SDS-PAGE Mini Gels

1. Clean and dry glass plates.
2. Assemble the plates and spacers using the thick plastic base and pre-attached clamps. Push down on everything before the clamps are tightened to be certain everything is flat and level to prevent leaks when pouring the acrylamide.
3. Snap the assembled plates into the casting stand where the gel will be poured.
4. Mix the appropriate amounts of resolving gel (12%) and pour between glass plates to fill two-thirds of the available space. Immediately pour approx 200 µL of water over the top of this acrylamide for an air-tight seal and let solidify (5–10 min; *see* **Note 7**).
5. Drain off the water and wipe away excess with a chemwipe. Mix and pour the stacking gel (4%), add the combs and let solidify.
6. Remove combs and rinse wells with dH_2O. Then snap plates containing solidified acrylamide into the running tank apparatus.
7. Place in a gel box and pour in 1X PAGE buffer in both the top and bottom chambers.
8. Load samples (approx 5–20 µL/lane) and run at approx 100 V for 1 to 2 h.
9. Stain with Coomassie or electrotransfer to nitrocellulose for immunoblot analysis (*see* **Note 8**).

3.4.3. Electroblotting of SDS-PAGE Gels

1. After the SDS-PAGE gel is run, it is possible to run an immunoblot on the contents. To do this, the gel will have to be transferred to nitrocellulose.
2. Set up the blotting "cages" in blot buffer (sodium phosphate, 25 m*M*) containing trays. The cages are part of the Bio-Rad Transblotter apparatus. For proper orientation, be certain the black side of the blotting cage is face down in the buffer-filled tray.
3. Assemble the blot by adding a buffer-soaked fiber pad (scouring-type) to the black side of the cage followed by a sheet of Whatmann paper, the SDS-PAGE gel, nitrocellulose (cut to cover the entire gel), Whatmann paper and, finally, the other fiber pad. Be certain to remove all bubbles by rolling a pipet over each layer.
4. Close the blot cage and slide it into the Bio-Rad electroblotting container with the black side of the blot cage facing the black side of the electrobotter. The electroblot chamber should be filled with blot buffer.

5. If needed, assemble the second blot sandwich in the same manner. Add it to the same electroblot chamber.
6. Cover the chamber and supply current at 0.9 A for 1.5 h. The electroblotting chamber should be kept cool using circulating water or sitting in an ice bath. Retrieve the blotted nitrocellulose filter from the chamber.
7. Put the membrane in a Petri plate or tray, add enough blot block to cover the nitrocellulose, close the plate or tray, and place on the rocker platform at room temperature for anywhere from an hour to several hours (usually 1–2 h is sufficient for blocking). At this point, it can also be labeled with lane assignments, date, etc. and stored flat at 4°C until ready to probe.
8. Remove the blot block solution from the tray. If using several different antisera, mark each nitrocellulose membrane with a Sharpie. Put each membrane in a separate tray, add 5 to 10 mL of blot block to each piece and the specific antisera under investigation (usually 100 µL of sera/10 mL blot block).
9. Incubate on a rocker platform at room temperature for 2 h.
10. Carefully remove the membrane(s) from the tray and place in plastic trays containing blot wash solution. Wash three times for five minutes per wash (all on the rocker platform).
11. Put the washed membranes in fresh trays. At this point the procedure differs depending on whether the detection antibody is horseradish peroxidase (HRP) labeled or some alternative label. The following is a protocol for detection of HRP conjugated antibodies.
12. Add HRP-labeled anti-bovine-HRP or other appropriate secondary antibody diluted 1:20,000 in blot block solution.
13. Incubate on a rocker platform for 1 to 2 h at room temperature.
14. Perform three 5-min blot washes (with blot wash solution) to the membrane.
15. Do a final wash in 1X PBS for 5 min.
16. Drain away all the PBS solution and add 5 mL of SuperSignal solution A and 5 mL of SuperSignal solution B to a tray containing the membrane and rock the solution over the membrane for 1 min.
17. Remove the membrane and place it on a glass plate containing fluorescent tape for orientation on the developed film.
18. Wrap the membrane and glass plate with Saran wrap and expose to film for 5 to 60 s in a dark room (*see* **Note 9**). Develop film (*see* **Note 10**). Increase or decrease exposure time as needed.

Specific antigens identified in these experiments must be further tested before they can be incorporated into immunodiagnostic tests that can then be used to detect infected animals. However, this genomic screen is a powerful approach to quickly identify candidate diagnostic antigens.

4. Notes

1. Primer concentrations are at 5 p*M*. Up to 5% of dimethyl sulfoxide is beneficial in PCRs involving DNA template with high G+C content. PCR nucleotides were added to a final concentration of 200 µ*M*. Taq concentration is 5 units per microliter.

2. The MBP system appears to be superior to polyhistidine-tagged proteins for production of *M. paratuberculosis* proteins in *E. coli* because a higher percentage of mycobacterial proteins are successfully expressed using this system. However, cleaving off the MBP affinity tag is often not 100% efficient, and even trace amounts can enable antibodies to detect MBP because of its immunodominance. Therefore, it is important to always use MBP-lacZ (expressed from the parent pMAL-c2 plasmid) or other nonrelevant fusion protein as a control in any downstream experiments.
3. Whether amplified product is gel purified or the reaction mixture is simply Gene Clean depends on how clean an aliquot of the amplification reaction looks on the test gel. If more than the amplified band is present in the ethidium bromide stained gel, then consider optimizing the amplification reaction or purify the product of interest by excision from an agarose gel and isolating it from the gel slice using the Gene Clean kit.
4. A longer induction using IPTG (3–4 h) may sometimes result in an increased production of fusion protein.
5. The amount of resin used will depend on the amount of fusion protein produced. Generally, the amylose resin will bind 3 mg of fusion protein per milliliter of bed volume.
6. The maltose added to the column buffer has a higher affinity for the amylose resin, thereby displacing the MBP fusion protein to be collected in the column eluate.
7. The water overlay is critical to obtaining very even solidification of the resolving acrylaminde. The water overlay must be gently added, usually with a syringe attached to a needle bent at a 45-degree angle.
8. It is important to express and purify the MBP-LacZ alpha peptide produced from the parent pMAL-c2 vector because it will serve as a size standard on the Coomassie stained gels and also as a control in downstream applications. The MBP alone is 42 kDa in size and the LacZ alpha peptide is another 8 kDa, resulting in a 50-kDa fusion protein. This fusion protein is expressed to high levels in the described system and, therefore, it is an easy control experiment to troubleshoot technical problems in the expression and purification phases.
9. The exposure time will vary with the concentration of primary antibody used. If the antibody is high titer, shorter exposure times will be needed. If the antibody is of low titer, 1- and even 2-min exposures might be necessary.
10. If a high background is observed on immunoblots, it may be due to residual *E. coli* proteins. In this case, it may be beneficial to preadsorb sera with an *E. coli* extract. The extract is available commercially from Promega; however, you may want to prepare your own using the *E. coli* strain selected for these experiments. Mix the extract 1:1 with the sera and incubate at room temperature with slow rocking. Centrifuge out the immune complexes and use the supernatant as the primary antibody.

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15

Diagnosis of Q Fever Using Indirect Microimmunofluorescence

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Summary

A microimmunofluorescence technique for the diagnosis of Q fever is described. Although this method is useful for serological diagnosis of Q fever, some technical difficulties are associated with it. First, the test antigens must be produced by a cell culture method in a level-3 biohazard facility and, second, the antigen *Coxiella burnetii*, which is the causative agent of Q, is characterized by the presence of two phases. To obtain phase I antigen, mice must be inoculated with *C. burnetii*. The materials obtained from the spleens of the infected mice are then used for cell inoculation. After purification, the antigens of both phases are deposited and fixed on multiple-well microscope slides. The serially diluted sera to be tested are incubated on these slides, then rinsed and overlaid with anti-IgG, -IgM and/or IgA secondary antibodies. Finally, the slides are examined under a fluorescence microscope for presence of *C. burnetii*.

Key Words: Microimmunofluorescence; Q fever; *Coxiella burnetii*; antigen phase I; antigen phase II; indirect immunfluorescence assay.

1. Introduction

Q fever is a ubiquitous zoonosis caused by *Coxiella burnetii*, an obligate intracellular organism. Cattle, goats, and sheep are the primary reservoir of infection. The animals excrete the bacterium via urine, feces, milk, and amniotic fluids. After desiccation, the bacterium spreads via aerosols. The respiratory tract is the usual portal of entry.

In its acute form, Q fever is a mild, self-limited "flu-like" illness that is characterized clinically by sudden onset of malaise, headache, chills, pneumonitis, and hepatitis ***(1)***. Although reported as a rare complication of acute Q fever, endocarditis is the principal manifestation of chronic Q fever ***(2)***.

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This chapter describes a serological method for the detection of *C. burnetii* using an indirect microimmunofluorescence assay (MIA). Other serological tests, such as complement fixation and enzyme immunoassay, also have been described.

MIA depends on the variation in antigenic composition of *C. burnetii*. The virulent phase I organism is isolated from a natural environment or laboratory infected mammals, whereas the avirulent phase II organisms arise during successive subcultures of *C. burnetii* in embryonated hen eggs or cell culture systems. In essence, phase I and II *C. burnetii* represent *C. burnetii* having smooth and rough lipoploysaccharide cell membrane, respectively ***(3,4)***. This phase variation is associated with deletions in the genome that appear after successive subculture ***(5)***. Although only phase I organisms are virulent for humans, antiphase II antibodies are normally found in patients with chronic Q fever. In contrast, specific antiphase II antibodies predominate during acute Q fever. MIA uses both phase I and II antigens of *C. burnetii*. However, for screening purposes, phase II antigen alone is used. The preparation of the two phases of the antigen is the first step of the procedure, followed by the deposition of the antigens on slides and documentation of the evidence of the presence of IgM, IgG, and/or IgA in the sera using specific secondary antibodies conjugated with fluorescein isothiocyanate. Cut-off values proposed for the diagnosis of Q fever diagnosis ***(6)*** using the MIA method and interpretation of serological results obtained with a single serum sample are summarized in **Table 1**. IgA is used mainly to follow-up patients who have been treated for chronic Q fever.

2. Materials

2.1. Gimenez Staining

1. Gimenez buffer: 3.5 mL of 2 *M* NaH_2PO_4, 15.5 mL of 0.2 *M* NaH_2PO_4, 19 mL of distilled water.
2. Carbol fuschin preparation: dissolve 5 g of basic fuschin in 50 mL of 95% ethanol. Mix 11.25 g of phenol in 125 mL of distilled water at 37°C. Mix the two solutions in 650 mL of distilled water.
3. Diluted carbol fuschin preparation: Mix 2 mL of carbol fuschin with 5 mL of Gimenez buffer. This diluted solution can be stored for 2 d at room temperature but must be paper filtered before each use.
4. Malachite green (china green) oxalate preparation: dissolve 2 mg of oxalate malachite 0.8% in 250 mL of distilled water. This solution may be stored for 4 mo at room temperature.

2.2. Antigen Preparation

Antigen preparation is restricted to specialized laboratories as the procedure must be performed in biohazard safety level-3 laboratory.

2.2.1. Mice, Cells, and Bacteria

1. Balb/c mice.
2. *Coxiella burnetii* Nine Mile (ATCC VR 615).
3. L929 mouse fibroblasts.

2.2.2. Cell Culture

1. Eagle minimal essential medium (Biowhittaker/Cambrex) supplemented with 2 m*M* L-glutamine (Biowhittaker/Cambrex) and 4% fetal bovine serum (Gibco, Invitrogen).
2. Phosphate-buffered saline (PBS), pH 7.3, supplemented with 0.1% formaldehyde.
3. PBS, pH 7.3, supplemented with 25% sucrose.
4. 150-cm^2 Cell culture flask.
5. 75-cm^2 Cell culture flask.

2.3. Microimmunofluorescence

1. 30-Well microscope slides (Dynatech Laboratories Ltd.).
2. 96-Well microplates for dilution (Dutscher, France).
3. Drawing pen (one for each antigen, rinsed and dried after each use).
4. Acetone.
5. PBS, pH 7.3, supplemented with 3% nonfat powdered milk.
6. Rheumatoid factor adsorbant (RF-absorbent, Behringwerke AG, Marburg, Germany).
7. PBS, pH 7.3, supplemented with Tween-20 (1/1000).
8. Fluorescein isothiocyanate-conjugated goat anti-human IgG (dilution 1:400), IgM (dilution 1:200), and IgA (dilution 1:100; Fluoline, Biomerieux, Marcy l'étoile, France). These secondary antibodies are diluted with a mixture of PBS with 3% nonfat powdered milk and a drop of Evans blue dye (Sigma, St. Quentin Fallavier, France).
9. Slides mounting reagent (Fluoprep, Biomerieux).

3. Methods

3.1. Gimenez Staining

This staining technique ***(7)*** is used during antigen preparation described in the following sections.

1. Apply diluted carbol fushin on the methanol fixed bacteria for 2 min.
2. Wash with distilled water.
3. Apply malachite green oxalate twice incubating for 9 s each time.
4. Wash with distilled water.
5. Dry and examine under 1000× magnification using an optic microscope.

3.2. Antigen Preparation

Antigen preparation is restricted to specialized laboratories because all the procedures must be performed in biohazard safety level-3 laboratory.

3.2.1. *Phase II* C. burnetii

1. Grow phase II *C. burnetii* Nine Mile (ATCC VR 615) on confluent layers of L929 mouse fibroblasts in 150-cm^2 culture flasks containing minimal essential medium supplemented with 2 m*M* L-glutamine and 4% fetal bovine serum.
2. Examine Gimenez-stained preparations of the cells scraped from the bottoms of the flasks under light microscope to document the presence of the cells that are already infected. When 90% of the cells are infected, pellet the cells and supernatants contained in each of the 15 flasks by centrifugation (5000*g*, 15 min) and resuspend in 1 mL of PBS (pH 7.3) with 0.1% formaldehyde.
3. These suspensions are pooled and kept at 4°C overnight.
4. All further steps are conducted at 4°C.
5. Fragment intact cells are by sonication and remove cellular debris by two successive centrifugations (100*g*, 10 min each).
6. Centrifuge the supernatants at 6000*g*, for 30 min in 20 mL of PBS with 25% sucrose.
7. Wash the cell pellet three times in PBS (6000*g* for 10 min).
8. Resuspend the washed cell pellet is in the smallest possible volume of PBS and adjust to a concentration of 2 mg/mL as determined by spectrometer.
9. Freeze the suspension of bacteria at –20°C for further use. The bacterial suspension can be frozen and is viable for at least 1 yr.

3.2.2. *Phase I* C. burnetii

1. Inoculate four Balb/c mice intraperitoneally with 10^6 phase II *C. burnetii* Nine-Mile to reactivate phase I *C. burnetii*.
2. Ten days after inoculation, remove the spleen of each mouse aseptically, grind in 7.5 mL of minimum essential medium with 2 m*M* L-glutamine and 4% fetal bovine serum, and use to inoculate L929 cell monolayers taken in three 75-cm^2 culture flasks.
3. Propagate the bacteria in 150-cm^2 culture flasks.
4. The number of subcultures is monitored by Gimenez staining and MIA (*see* **Note 1**).
5. The sera for MIA are derived from patients with Q fever that have been tested positive only for Phase II antibodies. The cultures may be subcultured (usually three to four times) as long as 98% of the bacteria remain at Phase I.

3.3 Microimmunofluorescence Assay

1. Deposit the two antigens prepared as described in **Subheadings 3.2.1.** and **3.2.2.** at the two poles of each well of a 30-well microscope slide using the tip of a marker.
2. Air dry the slides before fixing in acetone for 10 min. Initially, the sera are first diluted twice 1:5-fold and then serially diluted (twofold dilutions initially ranging from 1:25 to 1:3200 and more if needed) in PBS with 3% nonfat powdered milk.
3. Sera are first adsorbed with IgG rheumatoid factor adsorbant for 15 min for the determination of IgM and IgA.

Table 1
Interpretation of Serological Results for Q-Fever

Phase I antibody titer		Phase II antibody titer	
IgG	IgM	IgG	Interpretation
≤100			Active Q fever improbable
≥200	$50		Acute Q fever (100% predictive)
		≥1:800	Chronic Q fever (98% predictive)
		≥1:1600	Chronic Q fever (100% predictive)

4. 100 µL of sera (dilution 1:5) and 100 µL of RF in 300 µL of PBS are reacted.
5. The 30-well microscope slides have three lines. The first, second, and third line is used or IgG, IgM IgA, respectively. A total of 30 µL of each serum sample, diluted as above, is aliquoted into each well placing the most concentrated sample at the far right corner of the plate.
6. Incubate the overlaid antigens in a moist chamber for 30 min at 37°C.
7. Wash the antigens in PBS–Tween (1/1000) followed by one wash in PBS and another in distilled water. On each occasion, the overlaid antigens are washed for 10 minutes.
8. Overlay the air dried complex with 30 µL of fluorescein isothiocyanate-conjugated goat anti-human IgG (dilution 1:400), IgM (dilution 1:200), and IgA (dilution 1:100).
9. Incubate, wash, and dry the antigen-antibody complexes as described in the preceding steps.
10. Mount the slides with three drops of Fluoprep and examine under a fluorescence microscope (magnification ×400; *see* **Note 2**).
11. Each slide incorporates the sera obtained in patients with (positive control) and without Q fever (negative control). The titre obtained for positive control must be equal to that obtained previously (*see* **Table 1**) and the negative control must be less than 1:25.

4. Notes

1. The production of phase I *C. burnetii* antigen is the major difficulty associated with this method. In an *in vitro* culture of *C. burnetii*, phase II antigen becomes predominant after several subcultures. However, the remaining phase I cells are sufficient to produce infection in mice (phase II cells are destroyed by the mouse immune system). Thus, after injection of phase II antigen in mice only phase I antigen is present in tissues especially the spleen. After the inoculation of crushed spleen tissue in cell culture, phase I bacteria multiply. However, because phase II bacteria grow more quickly, after four or five subcultures, Phase I bacteria are less than 98% and thus not appropriate for use as antigen for the detection of

antibodies to phase I. For these reasons, after inoculation of crushed spleen tissue in cell culture, the growth of *C. burnetii* is monitored by Gimenez staining to evaluate the growth of the bacteria and by immunofluorescence to evaluate the contamination by phase II bacteria. This contamination must not be higher than 2% of all bacteria.

2. Compared with those of phase II samples, the interpretation of the results of MIA performed on in phase I sera is difficult due to presence of high background fluorescence. To determine the correct titer, the difference in the brightness of fluorescence must be considered rather than its extinction.

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16

Macrophage Cell Cultures for Rapid Isolation of Intracellular Bacteria

The Mycobacterium bovis *Model*

Massimo Amadori, Marco Ritelli, Silvia Tagliabue, and Maria L. Pacciarini

Summary

Isolation of *Mycobacterium bovis* from suspected cases of bovine tuberculosis demands laborious and time-consuming procedures. Also, direct PCR procedures on tissue samples show poor sensitivity, whereas radiometric and fluorescence-based identification procedures demand high running costs and do not reduce the time needed for isolation to less than 10 to 15 d. Owing to the aforementioned obstacles, the human macrophage cell line THP-1 and other macrophage cell lines were investigated in experiments of *M. bovis* propagation and isolation from organ samples. Macrophage cells can support a high-titered propagation within 48 h of minute amounts of both BCG and fully pathogenic *M. bovis* strains from organ samples. A proper antibiotic mixture prevents contamination of cell cultures. A seminested PCR for tuberculosis complex-specific insertion sequence IS6110 revealed *M. bovis* infection in infected cells. The same result can be obtained by a flow cytometry assay for expression of *M. bovis* chaperonin 10. The reduced time for isolation and identification of *M. bovis* (48–72 h) and the consistency of the test results make the use of macrophage cell lines attractive and cost-effective for veterinary laboratories involved in surveillance of bovine tuberculosis.

Key Words: Intracellular bacteria; tuberculosis; *Mycobacterium bovis;* diagnosis; macrophage cells; PCR; flow cytometry.

1. Introduction

A few species of pathogenic bacteria are facultative or obligate intracellular organisms both in vivo and in vitro. This is of utmost importance with regard to bacterial genera endued with a notable pathogenic and zoonotic potential,

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such as *Salmonella*, *Listeria*, and *Mycobacterium*. One of the world's most successful pathogens, *Mycobacterium tuberculosis*, has the extraordinary ability to persist and even to replicate in the extremely hostile environment of the host macrophage, where most other pathogens perish ***(1)***. Although macrophage cells present quite harsh conditions to the entering pathogen, they also can offer unique advantages. They are long-living cells and thus provide a potential long-term habitat for the bacterial invader. In addition, these cells play a pivotal role in host defence against infection, primarily attributable to a vast array of mediators, thus providing the invader organism with a unique opportunity to manipulate the immune system to its own advantage. Pathogenic mycobacteria such as *M. tuberculosis*, *M. bovis*, *M. leprae*, and *M. avium* have evolved complex and effective mechanisms for survival, including the ability to resist lysosomal delivery by inhibition of phagosome–lysosome fusion ***(2)***. Mycobacterial phagosomes are thus characterized by the absence of lysosomal markers such as lysosomal-associated membrane proteins and mature lysosomal hydrolases ***(3–5)***. Phagosomes harboring mycobacteria also contain markers for the early endosomal pathway ***(6,7)***, have a reduced amount of the vacuolar proton-translocating adenosine triphosphatase, and retain the Trp-Asp (WD) repeat-containing protein coronin 1 that prevents fusion of phagosomes with lysosomes ***(8–11)***. *M. tuberculosis* can enter the macrophage through multiple receptors without adversely effecting its survival ***(12)***. In particular, Mycobacteria use the complement receptors 1 and 3, which do not trigger the oxidative burst ***(13,14)***. Other effector mechanisms include the production of catalase and superoxide dismutase, which are capable of degrading reactive oxygen intermediates ***(15)***. The tubercle bacilli have been reported to down-regulate interleukin-12 ***(16,17)***, interferon (IFN)-γ;-mediated activation of the macrophage ***(18)***, the IFN-γ;-induced gene *gamma.1* ***(19)***, host cell apoptosis ***(20)***, and major histocompatibility complex class II ***(21,22)***. The production of the proinflammatory cytokine tumor necrosis factor-α and the antimicrobial effector nitric oxide also is critical for controlling *M. tuberculosis* infection. Interestingly, avirulent mycobacterial species elicit a vigorous tumor necrosis factor-α and nitric oxide response in infected macrophages as opposed to pathogenic species, thus supporting the contention that suppression of these effector activities may be important for virulence ***(23)***.

All together, these highly specialized and evolved replication and survival strategies adopted by intracellular replicating pathogens often are associated with major difficulties in isolation on bacteriological media. Difficulties turn into total failure of the isolation procedures in cases such as *Mycobacterium leprae* and *Lawsonia intracellulare*, where the use of bacteriological media must be replaced by animal models and molecular procedures. For example, *Lawsonia intracellulare*, the etiological agent of porcine proliferative enter-

opathy, can only be grown in vitro using the rat enterocyte cell line IEC-18 ***(24,25)***. Importantly, a license for a live-attenuated oral *L. intracellulare* vaccine was issued in the United States (Enterisol Ileitis, Boehringer Ingelheim Vetmedica Inc., Ames, IA), implying that a methodology for large-scale propagation of *L. intracellulare* has been established successfully.

On the whole, the main concerns about isolation of Mycobacteria on bacteriological media refer to tuberculosis (TB)-complex mycobacteria, which demand complex and time-consuming procedures. This is of utmost importance in case of *M. bovis*. The isolation of this organism from suspected cases of bovine TB is a critical task for diagnostic laboratories because the demonstration of the etiological agent is a precise requirement in many countries for the enactment of the zoo-sanitary measures aimed at protecting human health and eradicating the disease. Isolation in bacteriological media is the "gold standard" to be adopted for any sound evaluation of new and refined diagnostic procedures for bovine TB ***(26,27)***. Isolation must be supported by proper *post-mortem* inspection protocols ***(28,29)*** to identify the most suitable samples for bacteriological examination. In Europe, animal welfare considerations (EC Directive 86/609) have restricted the use of laboratory animals so that isolation in bacteriological media is now the only procedure allowed in many countries.

After a preliminary Ziehl-Neelsen staining of suspected organ samples, bacteriological examination actually is compounded by several hurdles. Difficulties related to the correct choice of samples, cross-contamination among bovine carcasses, the timing of TB-specific lesions, the large prevalence of nonvisible lesions reactors, unsuitable decontamination procedures in the laboratory, and even illegal use of isoniazid on the farm often may jeopardize the isolation of *M. bovis*. The duration of the isolation procedures (often 2–3 wk in liquid media and as long as 8–10 wk) is also of major concern.

The molecular techniques for detection of TB-complex mycobacteria based on PCR ***(30,31)***, or on alternative amplification strategies such as the transcription-mediated amplification system ***(32)*** and ligase chain reaction ***(33)*** are rapid and easy to perform ***(34)***. However, on *postmortem* samples, they do not show the same sensitivity and reliability as isolation in bacteriological media ***(35–37)***. The unsatisfactory performance of the molecular techniques on tissue samples can be traced back to the DNA extraction procedure. This is compounded in fact by a combination of poor homogenization of lymph node samples, high lipid content of the bacterial cell wall, and a lower concentration of bacterial cells (as compared with bacteriological media). In addition, PCR may be affected adversely by blood residues in organ suspensions ***(38)***. A wide discrepancy also was shown between isolation on bacteriological media and PCR detection in samples ex vivo after experimental infection with *M. bovis* ***(39)***. Finally, co-infections of *M. bovis* and *M. avium* ***(39)*** also are diagnostic hurdles.

Alternative radiometric and fluorescence-based protocols for isolation of *M. bovis* in a shorter time span are expensive and often subsidiary to bacteriological procedures for *M. bovis* isolation ***(40)***. An extensive investigation into the BACTEC MGIT 960 system (Becton Dickinson Diagnostic Instruments) based on liquid media and widely validated on human clinical samples ***(41–43)*** was carried out. The aforementioned system was compared with isolation on solid media (Löwenstein-Jensen and Stonebrink media) from 617 samples collected from 2002 to 2004, including bovine lymph nodes, lymph nodes from wild boars, and organ samples from pigs, birds, and farmed fish. The sensitivity of the MGIT 960 system was much higher than that of solid media with regard to the *M. bovis* strains (all from bovine lymph nodes). A better performance also was shown for *M. avium* isolation but not for the demonstration of some atypical mycobacteria. This comparison showed the mean time for organism isolation for these procedures to be 12 d for MGIT 960 and 25 d for solid media. These data are in agreement with previous reports about isolation of TB-complex mycobacteria by the same system ***(41,44–46)***. However, we felt that the improvement achieved with the MGIT 960 system did not match completely the demand of the current TB control programs because of the delay of important decisions about breakdown and suspected herds put under restrictions. In fact, the enactment of most zoo-sanitary measures impinges on the demonstration of the disease agent in organ samples.

In the light of these issues, we developed rapid amplification of *M. bovis* before any detection procedure (PCR or other). We reasoned that such an amplification could be afforded by macrophages or macrophage cell lines, which are validated extensively in experimental studies as models for in vivo survival, growth, virulence, and drug resistance of TB-complex mycobacteria ***(47)***. Most of our experiments were conducted on the human THP-1 cell line because it had proved to be a powerful and reliable means for the amplification of *M. bovis* and amenable to a large array of laboratory procedures in studies on both *M. bovis* BCG and *M. tuberculosis* ***(23,48,49)***.

In this chapter, we report on the diagnostic potential of THP-1 ***(50)*** and other macrophage cells for isolation of *M. bovis* and its detection by means of PCR and/or flow cytometry for bacterial chaperonin (cpn) 10 ***(51)***.

2. Materials

2.1. Cell Lines

1. THP-1, human acute monocytic leukemia cell line (Centro Substrati Cellulari, Brescia, Italy, and American Type Culture Collection ATCC). These cells have Fc and C3b receptors and lack surface and cytoplasmic immunoglobulins; they stain positive for alfa-naphthyl butyrate esterase, produce lysozyme, and are phagocytic ***(52)***.

2. P3 88 D1 mouse-monocyte-macrophage and U937 human histiocytic lymphoma cell lines (American Type Culture Collection ATCC).
3. The Mono Mac 6 (MM6) human monocytic cell line (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany).

2.2. Cell Culture

1. RPMI 1640 medium with L-glutamine (Gibco-BRL).
2. Fetal calf serum (mycoplasma and BVD virus-free; Gibco-BRL).
3. Heat-inactivated fetal calf serum (as in **step 2**, treated for 30 min at 56°C).
4. 100 m*M* Sodium pyruvate MEM (Gibco-BRL).
5. MEM nonessential amino acids 100X (Gibco-BRL).
6. 50 m*M* 2-Mercaptoethanol (Gibco-BRL).
7. Antibiotics for cell propagation: bacitracin, colimycin, and neomycin (Gibco-BRL).
8. OPI-supplement (Sigma) containing oxalacetic acid, sodium pyruvate, and insulin.
9. Phorbol 12-myristate 13-acetate, PMA (Sigma; soluble in ethanol; photosensitive; store at –20°C).
10. Antibiotics for control of infection in cells: Bactec Panta Plus Kit (Becton Dickinson).

2.3. Mycobacterium bovis *Strains*

1. *M. bovis (*BCG Copenhagen, ATCC 27290) grown in Middlebrook 7H9 medium (Difco). A mid-log culture can be supplemented with 6% glycerol, frozen at –80°C in aliquots, and then titrated on plates of Middlebrook 7H10 medium (Difco).
2. Pathogenic *M. bovis* strains from lymph nodes of *M. bovis*-infected cattle with or without typical TB lesions. After isolation on solid Löwenstein–Jensen medium, the identification of *M. bovis* must be unambiguous in terms of microscopy and cultural characteristics. The strains, which are frozen in aliquots supplemented with 6% glycerol, can be titrated in terms of colony-forming units (CFUs) on solid Middlebrook 7H10 medium. The original, *M. bovis*-positive lymph node suspensions can be also stored at –80°C in aliquots after homogenization in saline.

2.4. DNA Extraction and PCR

1. QIAamp DNA Mini Kit for DNA isolation (Qiagen).
2. dNTP set 100 m*M* solution (Amersham Biosciences Europe).
3. AmpliTaq Gold (Applied Biosystems).
4. Agarose gels for electrophoresis (Euroclone).

2.5. Antibodies

1. Murine monoclonal antibody (MAb) SA-12 (α-GroES; Department of Microbiology, Colorado State University, Fort Collins, TX ***[53]***). This MAb (IgG2a, *k* chain) recognizes the 48 to 60 amino acid sequence of chaperonin 10 (cpn 10) of *M. tuberculosis* (M.t. cpn10 ***[54]***). This sequence is maintained in *M. bovis* ***(55)***.
2. An irrelevant isotype-matched murine MAb for control.

3. A commercial preparation of polyclonal human immunoglobulins (hIg; Globuman, Berna, Milan).
4. Fluorescein isothiacyanate (FITC)-conjugated, goat $F(Ab')_2$ anti-mouse IgG (H+L) antiserum (Southern Biotechnology Associates).

2.6. Flow Cytometry

1. Fetal calf serum (FCS; Gibco-BRL).
2. Saponin (Sigma).
3. Paraformaldehyde (PFA; Sigma).
4. Sodium azide (Sigma).
5. Bryte-HS Flow cytometer (Bio-Rad Laboratories).

3. Methods

3.1. Cell Cultures

1. Maintain THP-1 cells as suspended cells in 175-cm^2 tissue culture flasks in RPMI 1640 medium, antibiotics, 0.05 m*M* 2-mercaptoethanol, and 10% FCS at 37°C in a 5% CO_2 humidified incubator.
2. Grow to a density of 0.8 –1x10^6 cells/ml and passage every 2–3 d.
3. Prior to use, stimulate THP-1 cells in 25 cm^2 flasks with 20 nM phorbol 12-myristate 13-acetate (PMA) at 37°C overnight, so as to stop proliferation and to allow the cells to adhere and to express a macrophage-like phenotype ***(48)***.
4. Culture P388 D1 cells in RPMI 1640 medium, antibiotic, plus 10% FCS at 37°C in a 5% CO_2 humidified incubator. These are used for the isolation procedure 1 d after the passage of cells at high concentration (about 5×10^5 cells/mL).
5. Before use, inspect the flasks for a layer of adherent cells. When cultures grow to high density, large numbers of cells can be easily flushed from the surface for further propagation.
6. Propagate Human MM6 cells in RPMI 1640 medium, antibiotics, 10% FCS, non-essential amino acids and OPI supplement at 37°C in a 5% CO_2 humidified incubator.
7. Culture U937 cells in RPMI 1640 medium, antibiotics, 10% FCS, plus 1 m*M* sodium pyruvate.
8. Before use treat cells with 4 n*M* PMA for 72 h at 37°C to induce differentiation into adherent, macrophage-like cells.

3.2. Infection of Macrophage Cells

1. Supplement homogenized organ suspensions in saline (10%) with a selected mixture of antibiotics at 1:40 final dilution (*see* **Note 1**).
2. Incubate Panta-treated organ suspensions at 4°C overnight.
3. Wash monolayers of PMA-treated, adherent THP-1 cells in 25-cm^2 flasks with RPMI 1640 medium without serum and antibiotics twice.
4. Add 0.25 mL of an organ suspension plus 0.75 mL of infection medium (RPMI 1640 medium, 10% heat-inactivated FCS, 2.5% Panta Plus) to each cell mono-

layer and incubate for 2 h at 37°C in a 5% CO_2 humidified incubator. Keep noninoculated control cells under the same conditions in infection medium.
5. After 2 h of adsorption, wash cells twice with serum and antibiotic-free RPMI 1640 medium and layer with 10 ml infection medium.
6. For studies with *M. bovis* laboratory strains, infect THP-1, P388 D1, and U937 cells under the aforementioned conditions at different multiplicities of infection with both BCG and pathogenic *M. bovis* strains.
7. For nonadherent MM6 cells, wash three times in serum and antibiotic-free medium and resuspend in infection medium at 2×10^6/mL.
8. Incubate each sample diluted in infection medium with 2 mL of cell suspension for 3 h at 37°C.
9. Wash MM6 cells three times in serum and antibiotic-free medium, resuspend in 10 mL of infection medium and incubate at 37°C for 48 h in 25-cm^2 flasks in a 5% CO_2 humidified incubator (*see* **Notes 2** and **3**).

3.3. Validation of the Procedure

1. For validation purposes, carry out the infection procedure with titrated aliquots of *M. bovis* (BCG and pathogenic strains). Whole cells can be checked after 24 to 72 h for the presence of acid-fast bacteria by Ziehl-Neelsen staining and PCR for IS 6110 (*see* **Suhbeading 3.4.**).
2. The efficiency of the test system can be evaluated by plating intra and extracellular *M. bovis* (*see* **Note 4**). Collect the cell supernatant at different times after infection and pellet detached cells at low centrifugal force (300*g* for 10 min).
3. Treat these and the adherent cells of a 25-cm^2 flask with 1 mL of 0.07% sodium dodecyl sulfate for 30 min at 37°C, followed by 1 mL of 6% bovine serum albumin for neutralization.
4. Prepare serial 10-fold dilutions of supernatant and cell lysate in saline plus 0.025% Tween-80.
5. Plate 100 µL of diluted samples on solid Middlebrook 7H10 medium.
6. Discard plates where bacterial colonies are present at day 5 of the experiment (contamination).
7. Count *M. bovis* colonies after a 2- or 3-wk incubation at 37°C in a humidified 5% CO_2 incubator.
8. Carefully evaluate the logistics and the infrastructure of the laboratory before implementing the test procedure (*see* **Notes 5** and **6**).

3.4. DNA Extraction and PCR

1. Extract bacterial DNA from macrophage cells 48 h after infection using the QIAamp DNA Mini Kit according to the manufacturer's directions.
2. Amplify a 200-bp sequence of the TB-complex-specific insertion sequence IS6110 ***(56)*** by the following seminested PCR protocol.
3. Add 5 µL of purified DNA to each reaction tube. The composition of the PCR mixture for the first step (20 µL) is 50 m*M* KCl, 10 m*M* Tris-HCl, pH 8.3, 1.5 m*M*

$MgCl_2$, 200 µ*M* (each) dNTP, 0.2 µL each primer (primers IS6110 Ext-1 [5´-CCCGGACAGGCCGAGTTT-3´] and Ext-2A [5´-CCGGCATGTCCGGGACT--3´]) and 1.25 U Taq polymerase in this first round.

4. Incubate the mixture for 5 min at 95°C, followed by 30 cycles of amplification: 30 s at 95°C, 30 s at 68°C, and 60 s at 72°C. This is followed by 3 min of final extension at 72°C.
5. Add 80 µL of a second reaction mixture, which differs from the first one by inclusion of primer Ext-1 in conjunction with the internal primer Int-1 (5´-CCC CATCGACCTACTACG-3´), to 20 µL of first round PCR product.
6. Incubate the resulting 100-µL mixture for 5 min at 95°C and submit to 40 cycles of amplification (30 s at 95°C, 30 s at 50°C, and 60 s at 72°C), followed by 5 min of final extension at 72°C.
7. In each round of amplification, run a positive and negative DNA control under the same conditions.
8. The final amplification product (200 bp) is analyzed on a 2% agarose gel at 100 V for 45 min with ethidium bromide staining (*see* **Note 7**).

3.5. Staining With MAb and Use of Flow Cytometry

1. Collect THP-1 detached cells by centrifugation and use standard trypsin treatment to collect attached cells. Fix cells in PBS–4% PFA ***(57)***.
2. Wash cells in ice-cold PBS–10% FCS–-0.1% saponin (PBS-FCS-S) and incubate for 10 min ***(57)***.
3. Combine 5×10^5 cells in a final volume of 50 µL with an appropriate dilution of MAb SA-12 (*see* **Note 8**) in PBS-FCS-S or PBS-FCS-S with buffer only, for 30 min at 4°C.
4. Wash cells twice and resuspend in 50 µL of PBS-FC-S containing a proper dilution of human-adsorbed, FITC-conjugated, goat $F(Ab´)_2$ anti-mouse IgG (H+L) antiserum for 30 min at 4°C.
5. Wash three times in PBS.
6. Resuspend cells in PBS–4% PFA.
7. Analyze in flow cytometer.

Cells are gated by a combination of forward and large angle light scatter. Analyze at least 10,000 cells (*see* **Note 9**). The percentage of cpn 10-positive cells can be obtained in two ways. In the first method, background staining with MAb SA-12 in uninfected cells is subtracted according to the following scheme: (% binding with MAb SA-12 – % binding with conjugate only in infected cells) – (% binding with MAb SA-12 – % binding with conjugate only in control cells). In the second method, the control uninfected cells are not included. Treat two aliquots of infected cells with 0.5 mg/mL hIg for 30 min at 4°C (FcR blocking; *see* **Note 10**) before adding MAb SA-12 and an irrelevant, isotype-matched MAb, respectively. The percentage of cpn10-positive cells is obtained by subtracting background staining of infected cells with the irrelevant MAb.

4. Notes

1. In our experience, the Panta antibiotic supplement generally can control bacterial contamination in organ suspensions for 48 h with no adverse effect on cellular viability. Therefore, a convenient diagnostic approach could be envisaged for TB-suspected organ suspensions, whereby a part of the volume could be submitted to the usual decontamination procedures (e.g., NaOH treatment) for bacteriological examination and another aliquot submitted to an overnight antibiotic treatment at 4°C before seeding on susceptible macrophage cells. Note that gross particles of organ suspension sediment during overnight incubation at 4°C. The supernatant can be thus easily be aspirated and used in the cell culture test.
2. THP-1 cells were chosen because of the convenient cultural conditions and of their extensive characterization as suitable vehicle for amplification of TB-complex mycobacteria ***(48,49)***. THP-1 cells can be efficiently infected by suspensions of *M. bovis*-infected lymph nodes and are therefore suitable for primary isolation and subsequent detection of *M. bovis*.
3. Other macrophage cell lines (P 388 D 1, MM6, U937) can also be infected by *M. Bovis*. The use of MM6 cells may be more convenient because they can be readily infected in form of cell suspension. MM6 cells previously have been used ***(58)*** to investigate the intracellular replication and the molecular pathogenesis of Legionella infection within human monocytic host cells. MM6 cells represent a mature macrophage-like cell line that expresses phenotypic and functional properties of mature monocytes and does not need to be stimulated by phorbol esters or 1,25-dihydroxyvitamin D3. In addition to Legionella, MM6 cells were found to support the intracellular growth of *M. tuberculosis* and *Chlamydia pneumoniae*. Pending extensive comparison data relating to sensitivity of the above cell lines, the *M. bovis* detection procedure must obviously be validated for the cell line and the test protocol adopted in each laboratory.
4. After infection with 100 CFUs of *M. bovis* BCG, 10^7 THP-1 cells can give rise in 48 h to ≥100,000 intracellular CFUs and to ≥50,000 extracellular CFUs after plating on Middlebrook 7H10.
5. The method could be of potential in the culture of intracellular organisms in diagnostic bacteriology. Such a procedure should be considered whenever isolation on bacteriological media from field specimens is impossible, unreliable, or too complex and lengthy. In these cases, failure or delay in isolation is often in conflict with the fundamental requirement of prompt therapy or enactment of zoosanitary measures in the case of notifiable diseases.
6. A careful cost–benefit analysis should offset the aforementioned advantages of cell culture procedures against the actual diagnostic demands and the possibly higher costs in terms of infrastructure and training of personnel.
7. The best results for DNA extraction from THP-1 cells after 48 h of infection were achieved by recovery of detached cells by centrifugation, gentle trypsinization of attached cells, extensive washing of all cells together in culture medium without serum, followed by resuspension in 500 µL of PBS. Cells are then heat treated at 95°C for 15 min. DNA is extracted on 100 µL of this suspension, according to the

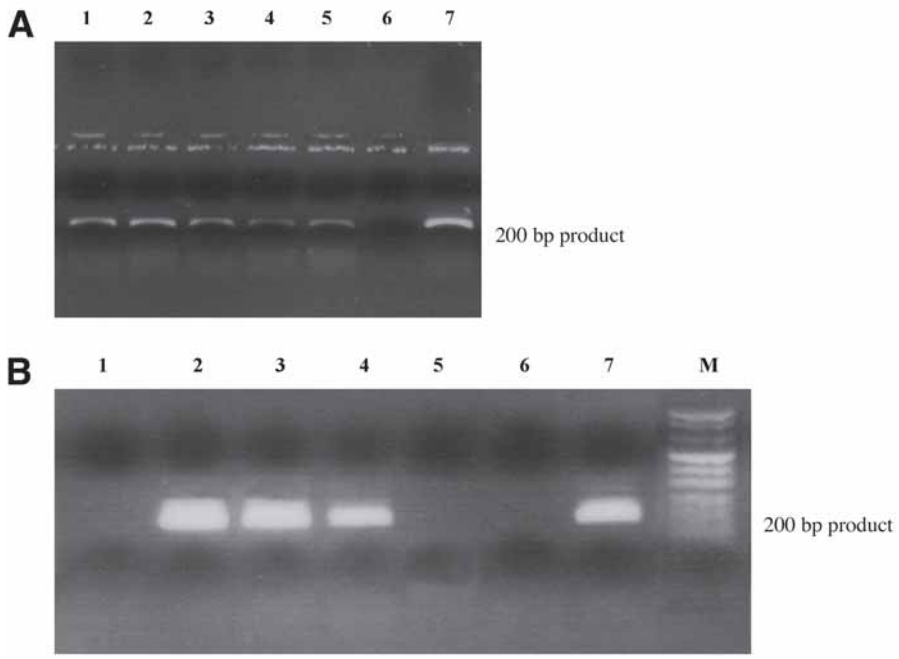

Fig. 1. **(A)** *M. bovis* strain 503 was grown in Middlebrook 7H9 medium. Serial 10-fold dilutions were used to infect THP-1 cells in 25-cm^2 flasks. Cells were lysed and submitted to seminested polymerase chain reaction (PCR) for IS 6110. Lane 1: cells infected with 15,000 colony-forming units (CFUs) of *M. bovis*. Lane 2: cells infected with 1500 CFUs of *M. bovis*. Lane 3: cells infected with 150 CFUs of *M. bovis.* Lane 4: cells infected with 15 CFUs of *M. bovis*. Lane 5: cells infected with 1.5 CFUs of *M. bovis.* Lane 6: PCR mixture without extracted DNA. Lane 7: positive control (DNA from *M. bovis* grown on solid Löwenstein–Jensen medium. **(B)** DNA was extracted from the aforementioned dilutions of *M. bovis* strain 503 and submitted to seminested PCR for IS 6110. Lane 1: PCR mixture without extracted DNA. Lane 2: 3000 CFUs of *M. bovis*. Lane 3: 300 CFUs of *M. bovis*. Lane 4: 30 CFUs of *M. bovis*. Lane 5: 3 CFUs of *M. bovis*. Lane 6: 0.3 CFUs of *M. bovis*. Lane 7: positive control (DNA from *M. bovis* grown on solid Löwenstein–Jensen medium), Lane 8 (M): molecular weight markers VIII (1114 to 30 bp, Roche).

"Tissue Protocol" of the kit, with the addition of 2 mg/mL (final) lysozyme to the lysis buffer. DNA extraction was unsuccessful on SDS-lysed cells. By this procedure, a positive amplification signal can be obtained after infection of cells with as few as 1.5 CFUs of a pathogenic *M. bovis* strain (*see* **Fig. 1**).

8. The use of MAb SA-12 for detection of cpn10 was prompted by the reasonable specificity of its target sequence for TB-complex mycobacteria (*M. tuberculosis* and *M. bovis*) and, in particular, by the lack of any crossreaction with the human

mammalian homolog of cpn10 ***(59)***. MAb SA-12 may crossreact with a restricted number of additional mycobacteria ***(60)***. However, this aspect would be of minor importance in case of bovine carcasses showing overt TB lesions. A further confirmation of *M. bovis* infection could be recommended instead in cases of bovine carcasses without visible TB lesions. Interestingly, direct examination of TB-complex mycobacteria also is possible by means of the Bryte HS flow cytometer ***(61)***. For this purpose, the MPB83 protein of *M. bovis* BCG was shown to be available on the cell surface for reaction with MAb MBS43 ***(62)***.

9. The highest expression of *M. bovis* cpn10 at 48 h can be observed after infection with as little as 10 CFU of *M. bovis* BCG. Expression of cpn10 is also clear after infection with *M. bovis*-positive lymph node suspensions.
10. The expression of Fc receptors in uninfected THP-1 cells varies as a function of the passage number. Furthermore, we surmised that different levels of FcR expression might occur after infection with *M. bovis*, thus affecting the level of unspecific binding of MAb SA-12. Therefore, we set up a further protocol of FcR blocking by pre-incubation with polyclonal hIg. The expression of *M. bovis* cpn 10 can be conveniently detected under these conditions, too. The cut-off value in the second method described may vary as a function of the cells counted. Theoretically with 10,000 infected cells stained with the relevant and irrelevant MAb, respectively, even a 1% difference could be highly significant (Fisher's exact *p* test), in the presence of optimal MAb dilutions. A 5% difference would be undoubtedly a more acceptable and robust threshold.

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