CHAPTER 1

PCR Basics

Amanda Fairchild[1], M.S., Margie D. Lee[1,2] DVM, Ph.D.,
and John J. Maurer[1,2*], Ph.D.
Poultry Diagnostic & Research Center[1], College of Veterinary Medicine,
The University of Georgia, Athens, GA 30602
Center for Food Safety[2], College of Agriculture and Environmental Sciences,
The University of Georgia, Griffin, GA 30223

INTRODUCTION

The safety of your food supply is an important goal of the U.S. government and diagnostic food microbiologists across the country. Up to 5,000 deaths and 76 million illnesses in the U.S. each year are associated with the consumption of foods laced with pathogenic bacteria (53), costing the U.S. an estimated $6.5–$34.9 billion annually (8). Even though bacteria have been shown to be the cause of the majority of food-related illnesses, the government does not have a mechanism for detecting and accounting for the losses due to other common foodborne pathogens, such as viruses and protozoa. Detection, identification, and quantification of foodborne pathogens are often made difficult by the low numbers of pathogenic organisms and interference from the food matrix that is being sampled. Bacterial pathogens of particular importance include *Listeria*, *Campylobacter*, *Escherichia coli,* and *Salmonella* (53), and the norovirus and

*Corresponding author. Phone: (706) 542-5071; FAX: (706) 542-5630; e-mail: jmaurer@vet.uga.edu.

hepatitis A virus are currently regarded as important foodborne viruses (44). However, since the advent of the polymerase chain reaction, finding these few pathogenic microorganisms in otherwise innocent looking provisions is becoming easier, mainstreamed, and second nature to many diagnostic laboratories. The polymerase chain reaction (PCR) is a simple way to quickly amplify specific sequences of target DNA from indicator organisms to an amount that can be viewed by the human eye with a variety of detection devices. A goal of the present-day food microbiology research laboratory is to use the growing database of bacterial genomic information, made available by researchers mapping unique identifier genes of foodborne pathogens, to design monitoring systems capable of analyzing various incoming samples for hundreds of different organisms accurately and efficiently.

Using Molecular Methods to Identify Microbial Pathogens. Prior to the 1980s and the advent of PCR, identification of microbial pathogens relied on bacteriological methods to enrich and isolate the organism from clinical or food sample, and subsequent biochemical and/or immunological tests to confirm the microbe's identity. During the past 30 years, we have gained tremendous insights into how microorganisms spread and cause disease. In several instances, pathology associated with many bacterial illnesses is attributed to a single gene (4, 9, 18, 75, 97). Other pathogens like *Salmonella* are more complex, requiring coordinate regulation of several virulence gene sets to cause disease (49). Therefore, the organism's genetics or genotype dictates its ability to cause disease, or the severity of the illnesses associated with it. Many of these virulence genes are unique to the pathogen and subsequently make useful markers for identifying said pathogen (37, 41, 86, 95). With several bacterial genomes completed, we now know the genetic basis for phenotypes that have been useful markers for distinguishing pathogens from closely related commensals that inhabit the same niche. By identifying gene(s) associated with phenotype (e.g., O157 serotype), we have identified a marker with greater specificity than afforded by the actual antigen itself, especially when confronting cross-reactivity and false positive associated with the immunological test (50). Although quite specific, the early molecular-based method, DNA: DNA hybridization had limited utility due to its limited sensitivity, time length, or safety issues associated with the use of radioactive probes (77, 80). Even with the introduction of nonradiometric methods for detecting hybridization of probe to its target gene, there was still the limitation of sensitivity, [i.e., the ability to detect the fewest cells possible (80)]. *How could one amplify the target gene enough to detect its presence in the sample contaminated with organism X?*

THE THEORY BEHIND PCR

The concept of the PCR was first described by Panet and Khorana in 1974(64) and owes its name to Dr. Kary Mullis and colleagues, who developed the process over the course of 4 months in 1983 at the Cetus Corporation. While driving down Highway 128 in Mendocino County, California, Dr. Mullis let his mind

slip back to the lab and a burning question that could not escape his mind. How could someone go about reading the sequence known as DNA, the language of our genes and blueprint for our existence? Like perfectly crafted ball and socket joints, the oligonucleotide base pairs within the DNA molecule bond to one another as the entire length of the ladder-shaped molecule twists into a corkscrew shape. Dr. Mullis referred to DNA structure as something like a mass of "unwound and tangled audio tape on the floor of the car in the dark" (58). In 1953, Drs. Watson and Crick had mentioned the biological significance of the DNA molecule with its complementary base pairing that suggested "a possible copying mechanism" for genetic material (91). One strand of DNA could be a template for the formation of a new complementary chain, and in the end you could have two DNA ladders, identical in every way. Dr. Mullis' development of PCR has extrapolated the copy machine theory one step further. He stated this simply as an analogy to a "'Find' sequence in a computer search" (58). This technique would have equivalent power to the latest computer displaying results of finding a document that consisted of just one word taking up 20 kilobytes of space on a hard drive the size of 150 Gigabytes littered with files of different types and sizes; like finding the code for blue eyes—and that code only—within the code that sums up every single trait for a person.

The second aspect Dr. Mullis had to account for in this process would be the ability of the chemical program to display the located sequence in a large enough fashion to be detected by the human eye. Dr. Mullis knew that if he could produce a short piece of DNA to find a sequence flanking a gene of interest and then start a process that could make the sequence reproduce itself over and over (hence a chain reaction), the concept of PCR could be realized. After all, it was already known that DNA innately makes a copy of itself when cells divide, so that each daughter cell can have a copy. If he introduced his "find" search strings, or primers, into a tube with DNA encouraged to uncoil from its natural double helix by heating and a biological glue (polymerase) to attach deoxynucleoside triphosphates (dNTPs) to the freshly uncoiled DNA at the location, where the primers bonded, a new copy of the DNA would be produced as specified by the primers that included the gene(s) of interest as the temperature decreased. The DNA polymerase that is used in some PCR reactions is made from the bacteria *Thermus aquaticus*, which was found originally in Yellowstone thermal water reservoirs. It is stable in temperatures above that which denatures DNA, making it a perfect enzyme for the job of attaching free dNTPs to make a new strand of DNA. There are other polymerases available that can actually proofread the addition of dNTPs, so there will be no errors made in the synthesis of longer PCR products. After each cycle in a PCR assay, the amount of DNA present doubles, so repeat the cycle and there are 4 copies of the gene, repeat again and there are 8 copies. With 30 cycles of this process, there would theoretically be just over a billion copies of the sequence in question (2^{30} = 1.07 billion). Find a way to tag each copy to make it visible to the human eye, with more copies making a stronger visible signal and you have proof of the presence of the small sequence embedded within the large DNA molecule you started with. A widely used method for viewing PCR products

involves running them on an agarose gel, staining the gel with ethidium bromide, and observing and photographing the gel on ultraviolet (UV) light source (56). The process is relatively fast, dictated by the amount of time it takes to heat the DNA strands until they will separate, the time to reduce the temperature so the primers bind to the single-stranded DNA, and the time allowed for the polymerase to add individual deoxynucleoside triphosphates to extend the forming DNA molecule. Dr. Mullis stated that scientists claimed that PCR made DNA research boring (57). Even though PCR is often considered "cookbook chemistry" because of its simplicity, his suggestion could not be further from the truth. For example, PCR has been one of the most important genetic tools available to those mapping the human genome and for those attempting to detect pathogenic bacteria, viruses, and protozoa. The PCR has made its way from the research lab to forensic and diagnostic laboratories worldwide. There have been considerable efforts to validate and standardize this tool (17, 38); to become a normal routine task/service performed by reference laboratories (3, 48, 76), and clinical diagnostic and food microbiology laboratories (1, 33, 38, 45, 54, 79, 87).

THERMOCYCLER TECHNOLOGY

Since the technique of PCR was developed, there have been many evolutions of the equipment that makes the process possible, based on the concept that strands of DNA denature, or unwind, and anneal, or wind again back into the helical corkscrew, in response to fluctuations in temperature. The first successful PCR reaction took place using water baths at the appropriate temperatures for each step in the procedure, with the technician moving vials by hand from bath to bath at the appointed time, for 30 or more cycles to get adequate amounts of DNA copies that could be detected. Nowadays, thanks to automation, PCR reactions can be set up in thermocyclers that over the course of minutes to a few hours reliably yield high numbers of a specific DNA sequence if present in a sample.

The standard thermocycler uses a large heating block, into which microcentrifuge tubes are placed. This type of thermocycler through its computer controls the heating and coolings of the blocks through the cycles of each reaction. Sometimes an oil or wax overlay is put on the samples within the microcentrifuge tubes to keep the sample from escaping the bottom of the tube during the heating for the denature step of the PCR reaction. This type of machine is not as desirable because of the time it takes to heat and then cool the entire block to the appropriate temperature within each cycle. The time required for the heat block to uniformly reach each temperature coupled with the slow heat transfer rates to the microcentrifuge tubes makes this type of thermocycler virtually inadequate with today's demand for high-speed accurate amplification of PCR products.

The RapidCycler, manufactured by Idaho Technologies, is an example of equipment designed to provide the quick temperature cycling necessary for

PCR reactions. This type of thermocycler uses heat transfer through blasts of high-velocity hot air to accomplish the temperature transactions from the initial heating of the DNA sample through the annealing of the primers and the extension of the new double strand of DNA by the polymerase. There is overall temperature uniformity within the cavity of the reaction vessel and rapid heat exchange within the sample because individual samples are loaded into micro-capillary tubes or thin walled microcentrifuge tubes for the reactions to take place. This also allows for a smaller overall volume in each reaction tube, thus saving valuable amounts of reaction components such as polymerase and primers. After the PCR cycles are complete, the samples are loaded on an agarose gel that contains ethidium bromide, and viewed under a UV light source.

A gradient thermocycler allows the clinician to optimize each of the three temperatures needed for the denaturing, annealing and extension of new DNA products. Optimization might be required if an existing PCR cycle program cannot be located for detection of a particular gene sequence. Optimizing the PCR reactions is critical to the success of the production of amplicons and is not always the easiest thing to do. The melting temperature can be calculated for the primers when they are made, but the denaturing and annealing temperature of the cycle might have to be determined by educated guess with some trial and error, possibly rerunning the same reaction with many different temperatures before the best fit temperature is found. Luckily, most machines have the ability to reach the different temperatures at the same time (thus the gradient), so the reactions are run at the same time with results from each temperature trial collected at the same time. The block used to hold the samples in this type of machine can be programmed to heat over a gradient of about 20°C range with the annealing temperature for the PCR reactions increased by an increment of 1 or 2°C.

Another thermocycler type offers PCR product detection at the same time as each cycle of the PCR reaction progresses. It allows the technician to track

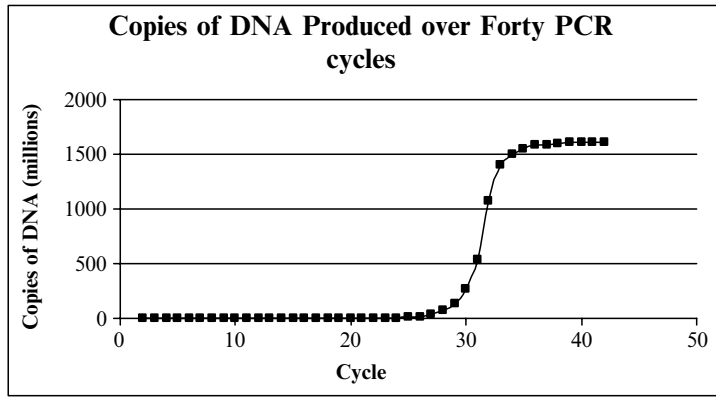

Figure 1.1. Example of real-time PCR output graph showing amount of DNA sequences produced over 40 PCR cycles.

the increase in products during a PCR reaction as displayed on a graph (Fig. 1.1). Higuchi and colleagues introduced this feature, dubbed "Real-Time" PCR, and described how the number of cycles necessary to produce a detectable fluorescence was "directly related to the starting number of DNA copies" in a sample (32). There are a number of companies that offer this technology, which combines the rapid cycle polymerase chain reaction with fluorescent detection of amplified PCR product in the same closed vessel as the reaction mix. The primers are usually labeled with fluorogenic probes, or a DNA-binding dye is included in the PCR reaction, which fluoresces under light emitted at a certain wavelength.

DETECTION

Detection of the PCR product or "amplicon" can be accomplished several ways. Following PCR, the sample is loaded into an agarose gel, and the DNA fragment(s) or amplicon if present in the sample is separated by electrophoresis based on size. Molecular weight, DNA standards are included to estimate size of amplicon(s) present in positive samples and positive control. The agarose gel and electrophoresis buffer contain a dye, ethidium bromide that binds double-stranded DNA and fluoresces upon excitation with UV light. This dye is used to visualize the DNA in an agarose gel. As the primers bind to fixed position within the target sequence, the expected size for our PCR product/amplicon is the distance between the forward and reverse primers. For example, if forward and reverse primers bind target gene X at positions 850 and 1000, respectively, then one expects to observe an amplicon of 150 bp for positive control or any sample that bears organism that contains gene X. The size of the amplicon is extrapolated from the DNA standards included in the gel. The sample MUST produce an amplicon of expected size predicted for the primers used and corrobated by the positive control before it can be considered positive by PCR. There is an inverse linear correlation between the $\log_{10}$ size of the DNA fragment (bp) and the distance migrated by the DNA fragment in the agaraose gel. The smaller the DNA fragment the farther it migrates through the agarose gel during electrophoresis. Therefore one can estimate the amplicon's size from DNA standards included with the agarose gel. As most PCRs produce small size amplicons (100–1,000 bp), one must use DNA standards that accommodate this size range and agaraose concentration (1.5%) that resolves small DNA fragments sufficiently to accurately determine the size for DNA band X. The PCR result is recorded photographically with a polaorid or digital camera with the appropriate lens filters and exposures for capturing images illuminated by the UV light.

Detection systems are slowly moving towards nongel methods for detecting and recording PCR results. Enzyme-linked immunosorbent assay (ELISA) has been developed for detecting amplicons (37). In the PCR reaction mix, the standard nucleotides have been substituted for chemically "tagged" nucleotides (e.g., dioxygenin or DIG). During PCR, the "DIG'-labeled nucleotides are

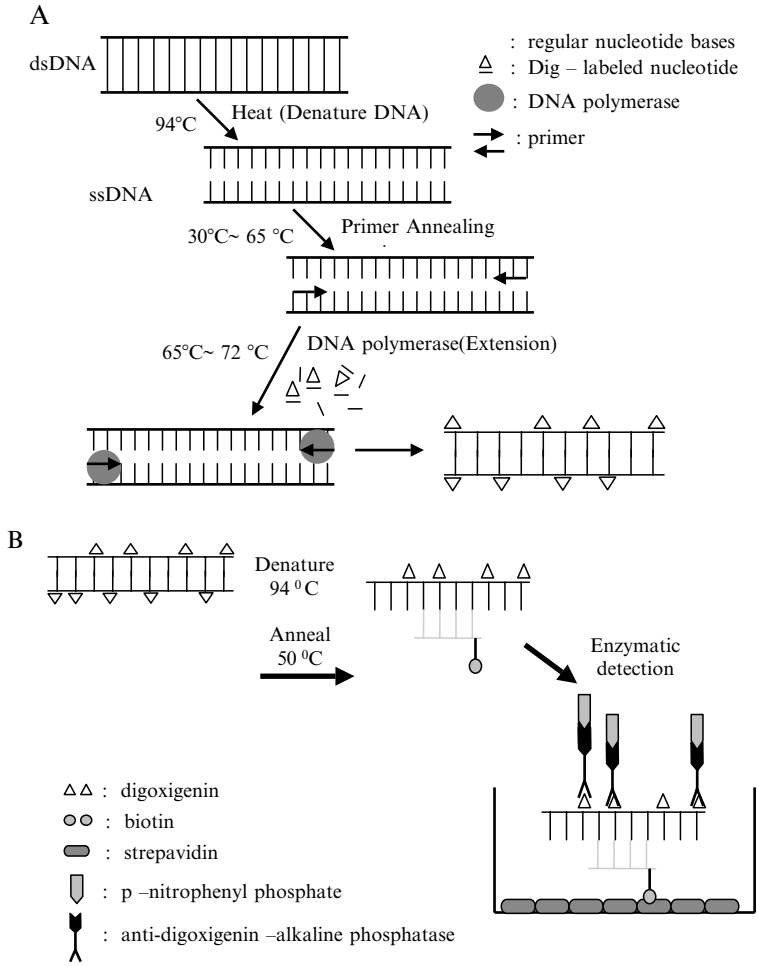

Figure 1.2. PCR-ELISA. Digoxygenin (DIG) labeled, nucleotides are incorporated into amplicon during PCR (A). An internal, 3′ biotinylated oligoprobe anneals to denatured, single-stranded amplicon following PCR. The strepavidin, coating the wells, binds to the biotin moiety of the oligoprobe and thus captures the amplicon. The amplicon is then detected using anti-DIG antibody enzyme conjugate (B). The oligoprobe adds additional specificity to this PCR test.

incorporated into the amplicon as it is synthesized (Fig. 1.2A). Following the last round of PCR, the sample is denatured and allowed to anneal with 5′, biotin-labeled, internal oligonucleotide. This oligoprobe binds to the complementary sequence present within the amplicon. This amplicon-oligoprobe hybrid is captured in strepavidin-coated 96 well microplate through the interaction of the biotin group with the strepavidin (Fig. 1.2B). The bound amplicon is visualized colormeterically using anti-DIG antibody enzyme conjugate, usually either horseradish peroxidase or alkaline phosphatase. The advantage of

this "PCR-ELISA" is that it easy to scale-up for high throughput of samples and lends itself quite well to automation. Another nongel method for detecting PCR amplicons involves detecting fluorescent dyes bound to or released from the amplicon using a fluorometer. This detection method is the basis of real-time PCR discussed below.

ADVANCED PCR TECHNOLOGIES AND MICROARRAYS

Real-Time PCR. Real-time PCR technology is based on the ability for the detection and quantification of PCR products, or amplicons, as the reaction cycles progress. Higuchi and colleagues introduced this technology (32) and it is made possible by the inclusion of a fluorescent dye that binds the amplicon as it is made (Fig. 1.3A). There are several ways to detect the PCR products under fluorescent detection. In TaqMan PCR, an intact, "internal" fluorogenic oligoprobe binds target DNA sequence, internal to the PCR primer binding sites. This oligprobe possesses a reporter dye that will fluoresce and a suppressor dye known as a quencher that prevents fluorescent activity via fluorescence resonance energy transfer (FRET). After each PCR cycle, when the double-stranded DNA products are made, a measure of fluorescence is taken after the fluorogenic probe is hydrolytically cleaved from the DNA structure by the exonuclease activity of the *Thermus aquaticus* DNA polymerase (29, 36). Once cleaved, the probe's fluorescent activity is no longer suppressed (Fig. 1.3B). FAM (6-carboxyfluorescein) and TAMRA (6-carboxy-tetramethyl-rhodamin) are most frequently used as reporter and as quencher, respectively. This PCR is often refered to as 5′exonuclease-based, real-time PCR or TaqMan PCR (55). When a DNA-binding dye is used, as more DNA copies are made with each successive cycle of the PCR, they are all bound, or intercalated, with the dye, and the fluorescence increases (Fig. 1.3A). SYBR Green I is the most frequently used DNA-binding dye in real-time PCR.

Two additional advanced methods of amplicon detection are hybridization probes and molecular beacons. The hybridization method uses fluorescence resonance energy transfer from one probe to another after annealing of the primers to the template strand of DNA. One probe has a donor dye at the 3′ end of the oligonucleotide and the other probe has an acceptor dye at the 5′ end. When both probes anneal to the target sequences, they are situated to have the dyes adjacent to one another one base apart. While in that configuration, the energy emitted by the donor dye excites the acceptor dye, which emits fluorescent light at a longer wavelength. The ratio between the two fluorescent emissions increases as the PCR progresses and is proportional to the amount of amplicons produced. Molecular beacons are short segments of single-stranded DNA. They use a hairpin shape to facilitate quenching of the fluorescent signal until the probe anneals to the complementary target DNA sequences, produced from PCR.

Some advantages of real-time technology include high sensitivity with the use of an appropriate probe or DNA-binding dye, ability for detection of relatively small numbers of target DNA copies, and ease of quantification because

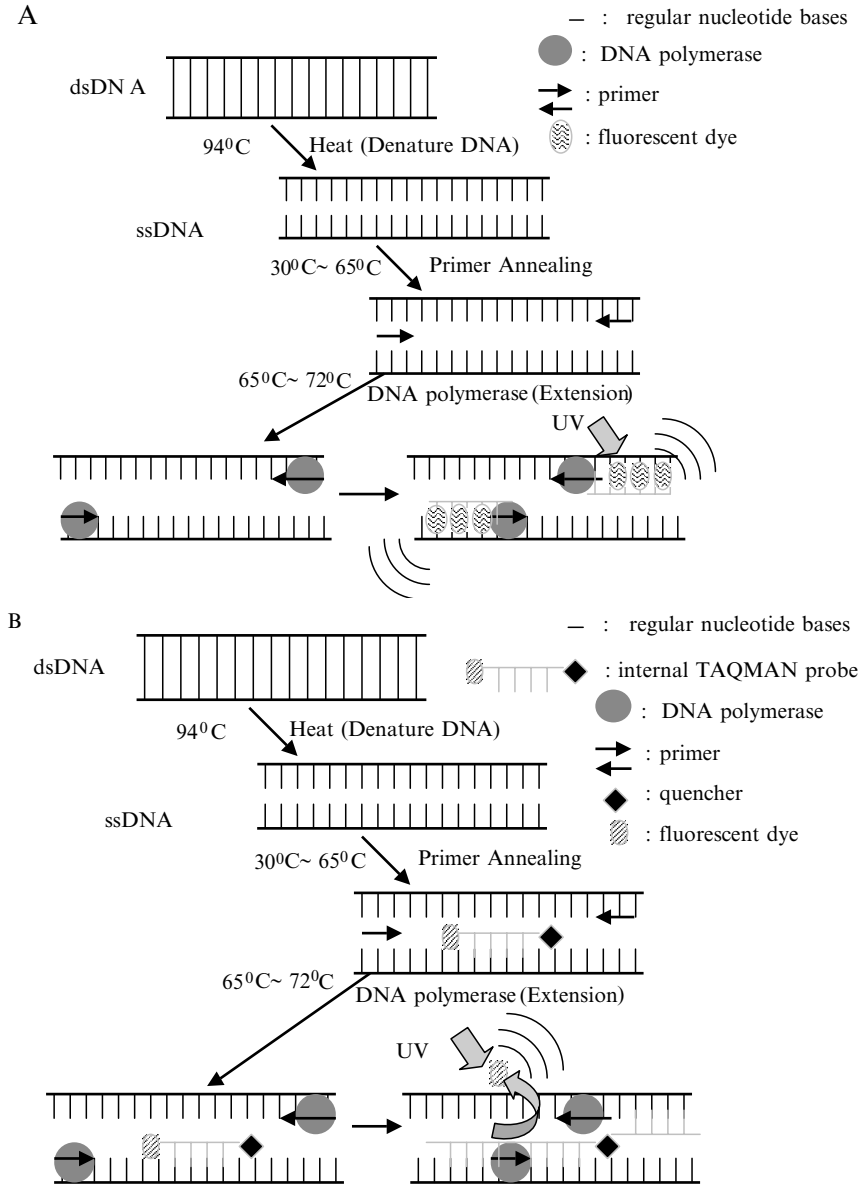

Figure 1.3. "Real-time" PCR detection of amplicons. Using fluorogenic dyes, amplicons can be detected by a fluorimeter as they are synthesized with each PCR cycle. Intially, a fluoresent dye, SYBR Green (A), was used to detect the amplicons. In this PCR, SYBR Green binds the double-stranded, DNA amplicon and fluorescences upon illumination with ultraviolet light (UV). Subsequently, real-time PCR was developed using an internal oligoprobe for detecting the amplicons. In TaqMan PCR (B), the oligoprobe contains a fluorscent marker and a chemical group that quenches fluorescence of the oligoprobe until the dye is liberated by 3′ exonuclease activity of the Taq DNA polymerase. This can only occur if the oligo binds the complementary sequences present in the target gene and amplicon.

of the lack of post-PCR detection measures. Molecular beacons can even be used to detect single nucleotide differences (27). Disadvantages of real-time PCR technology lie with the detection of the amplicons. If the DNA-binding dye is used, then any double-stranded product is labeled and fluoresces, including primer–dimers, and nonspecific amplicons, whether they are close to the target DNA in sequence or have erroneous secondary structure. The effectiveness of the fluorogenic probe is also influenced by the creation of primer–dimers, and both methods of detection are susceptible to less than optimal design of primers for use in the PCR and primer concentration in the master mix. To compensate for the unspecific binding of the DNA dye, real-time PCR equipment has the capability of running a melting curve after the PCR assay, which increases the temperature of the vessels in tiny increments until the fluorescence is lost due to the DNA denaturing. When the melting temperature of the target DNA sequence is reached, a sharp loss of fluorescence will be recorded. If additional losses are recorded, there may be PCR contamination, or the parameters of the PCR assay were not stringent enough, such as suboptimal primer design or temperature choice for the program. This feature of the equipment makes DNA-binding dyes a feasible and often cheaper alternative to the other methods available. The melting temperature of the amplicons should be known when designing primers for the assay and are usually referenced when programming the annealing step of the PCR reaction. Accurate primer design and optimization of the PCR reaction conditions for the primers are required in any PCR application, but especially with real-time technology.

Multiplex PCR. Multiplex PCR is a way to amplify two or more amplicons in a single PCR reaction. For multiplex PCR, each primer set is designed to its target gene to amplify a PCR product of a size unique to the target gene. To perform a multiplex PCR, the concentrations of primers, Mg^{2+}, free dNTPs and polymerase are altered to allow for the synthesis of the genes of interest, while the PCR reaction temperature parameters are optimized to the best average for amplicon production for all primer sets. This technique saves time and labor since more than one target DNA sequence is detected for each reaction, but might not be optimal if the PCR products are close in size and detection requires viewing an agarose gel stained with ethidium bromide. In a single PCR reaction, one can determine the identity of the organism (28, 39) or genotype (21), as the amplicon(s) size is unique to specific organism or gene. Therefore, it is possible to detect multiple pathogens in a sample from a single PCR test (65).

Terminal Restriction Fragment Length Polymorphisms (TRFLP). We can use PCR to characterize microbial communities and identify member species using a single PCR primer set. This PCR targets the 16S rDNA, a gene that is universally conserved among all bacterial species and amplifies a single ~1,500 bp amplicon. We can resolve diversity of 16S rDNA amplicons generated from this PCR using restriction enzyme(s) that recognize restriction sites within genus or species specific sites within this gene and produce DNA fragments, whose size corresponds to a specific genus or species (46). This PCR involves

using universal 16s rDNA primers in which one of the forward or reverse primers is fluorescently tagged (Fig. 1.4). Following PCR, the amplicons are digested with a restriction enzyme and subsequently loaded onto capillary bed of an automated DNA sequencer. This method has been refined and applied to automated DNA sequencers to resolve minor (x bp) differences between DNA fragments, monitoring and measuring fluorescence associated with the various sized DNA fragments as they elute from the sequencers' capillary bed (Fig. 1.4). Fluorescently labeled molecular weight standards are included to calibrate column in order to demarcate and identify the molecular weight for each DNA fragment separated by on the sequencer's capillary column. Each peak corresponds to specific genus/species present within the sample. The identity is determined from comparisons to an established database of restriction fragments predicted from 16S rDNA sequences (47). This database can be generated in house, from cloning and sequencing your 16S rDNA library or comparing it against an ever-expanding Web-based 16S rDNA database (Michigan State University Center for Microbial Ecology; *http://35.8.164.52/html/TAP-trflp.html*; 47). The latter has a tool for analyzing your TRFLP profile against this database, for various restriction enzymes. Depending on the restriction enzyme used, one may not be able to resolve various species or genera with a

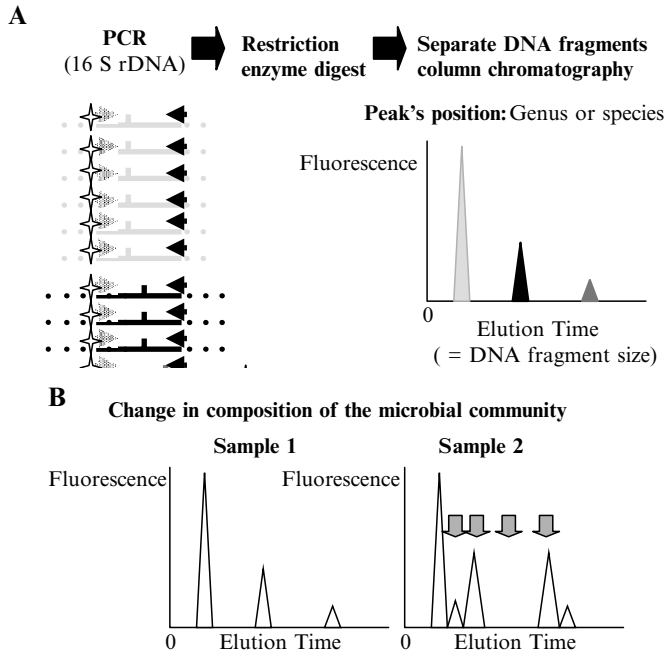

Figure 1.4. Characterizing microbial communities and identification of pathogens in foods from terminal restriction fragment length polymorphisms (TRFLP) of total microbial community 16S rDNA. (A) Concept behind TRFLP. (B) Interpretation of TRFLP.

single restriction enzyme. This is because they produce the same size DNA fragment with restriction enzyme X. It may take a number of different TRFLP profiles of the same community, generated with different restriction enzymes, before genera and/or species differences can be resolved (47). This method is currently used in assessing stability and structure of microbial consortiums, and it has been recently applied to analyzing changes in the community structure of gastrointestinal microflora in response to diet or probiotics (34, 42). TRFLP can also identify signature peaks for microbial pathogens (14, 60), where differences in 16S rDNA can be discerned between them and closely related commensal organisms, exceptions *E. coli* vs. *Salmonella* (61). Theoretically, TFLP and other molecular ecology tools (e.g., DGGE) will prove useful towards analyses of microbial communities present in foods, gastrointestinal tracts of food animals, probiotics and starter cultures and determine the impact certain food processes have on their composition, with regards to the food's safety for consumers.

Microarrays. Macroarrays, microarrays, high-density oligonucleotide arrays, and microelectronic arrays are all part of a new technology that allows one to screen for gene(s), sequence(s) or specific mRNA among myriad of possible sequences or genes in a single test (22). DNA hybridization arrays are based on specific positioning of a myriad of oligonucleotides or PCR amplicons, representative of a complete bacterial genome, on nylon membrane (macroarray), glass slide (microarray), or electronic microchip (microelectronic array). Each position on this solid support contains an oligonucleotide or PCR product unique to a particular gene. Total mRNA or genomic DNA from an organism is fluorescently or radioactively labeled and used in hybridization with solid support. The bound oligonucleotides or amplicons on the solid support serve to capture labeled probe in the RNA: DNA or DNA: DNA hybridization (Fig. 1.5). The labeled nucleic acid hybridizes to the position or "spot" on the solid support that contains complementary sequence for the labeled probe to bind. Identity of gene or sequence relates back to the original positioning of the oligonucleotides or amplicons on the solid support (Fig. 1.5). This technology has already been applied towards the study of bacterial gene expression (30, 71), host-microbe interactions (15, 73, 84), bacterial evolution and population genetics (6, 11, 23, 70, 85, 96). Currently, microarrays have been applied towards PCR-based detection of pathogens in the environment (2, 43, 65, 88). At present, this methodology is experimental, performed primarily by research laboratories. However, advancement in technologies and manufacturing will someday make microarrays affordable and practical for use in diagnostic setting, as PCR has now become.

DESIGN AND OPTIMIZATION OF DIAGNOSTIC PCR AS APPLICABLE TO FOOD MICROBIOLOGY

To perform PCR in any microbiological application, the DNA sequences of an infectious agent must be known, and the target sequences must be unique to the organism(s) to be detected. For example, if a food sample is suspected to be

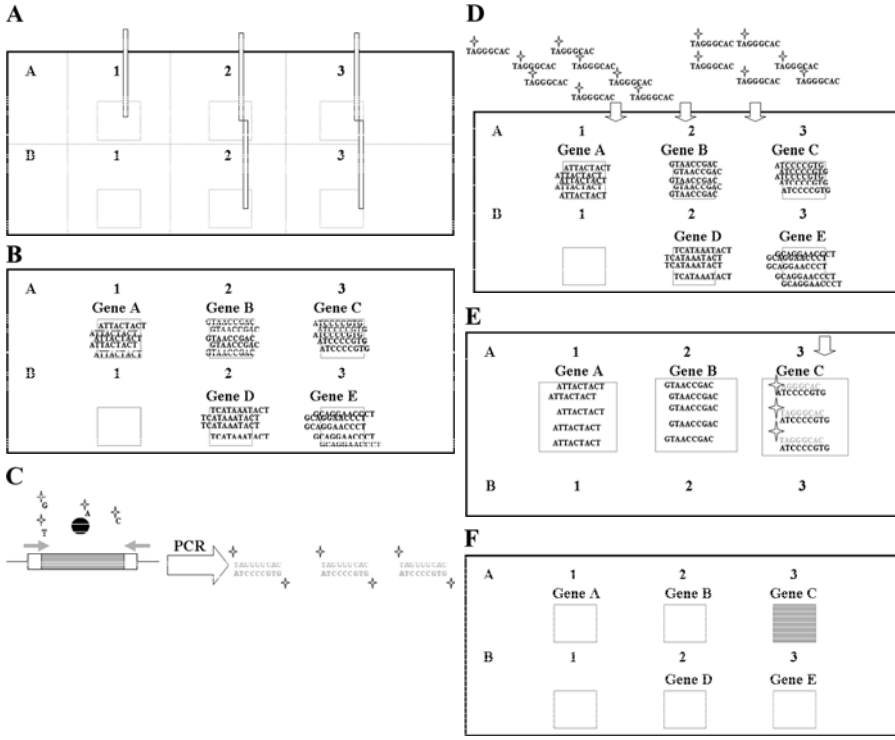

Figure 1.5. Microarrays. Specific oligonucleotides or PCR amplicons are spotted onto defined region on glass side or nylon membrane (A, B). The positioning of this capture probe on this solid matrix defines gene or signature sequence for organism X. If any of the genes present on slide or membrane are present, then it will be amplified during PCR and labeled with fluorescent nucleotide (C) and subsequently bound to the complementary sequence present on the solid support (D, E). Position of fluorescent signal (F) identifies gene or organism present in the sample.

contaminated with bacteria "X", such as *E. coli* O157, then a PCR can be used to determine the presence of the bacteria if there is a gene that only that bacteria possesses, such as an identifier gene "X1", or in the case of *E. coli*, the O157 antigen biosynthesis gene (50). If the gene was found in more than one bacteria type, say gene "XY4," additional PCRs would have to be performed to separate bacteria that harbor that gene (bacteria X and bacteria Y) by looking for a unique identifier gene of the target bacteria X, but at additional work, cost and time for the clinician. The case of identifying *E. coli* O157:H7 might require a multiplex PCR approach because of the closely similar genes of the different antigen subunit serotypes (21, 26). There are many genes that are shared within the same genus and species of bacteria, such as the genes shared among pathogenic *E. coli* strains. Instead of differentiating between bacteria X and Y, the researcher is met with finding a uniqueness of bacteria X1 versus bacteria X2.

Systematic Approach to Creating Your Own PCR. The development and validation of PCR is a long and arduous journey from concept to application. It involves identification of a candidate marker or allele for pathogen X, whose distribution among microbes is strongly associated with the pathogen in question, and the cloning and sequencing of the cognizant gene(s) associated with the marker or allele (50). For antigenic variable, surface proteins like flagellin, PCR, using primers that recognize conserved sequences flanking sequence variable regions (19, 81, 83), and subsequent sequencing of the PCR amplicon has identified sequences unique to serovar (26, 31) or pathogen (63), which subsequently led to development of serovar or pathogen-specific PCR (26, 31, 63). Design and development of PCR is the pursuit of researchers and if a PCR is available, commercially or otherwise, it is best to adapt this PCR to your lab than having to start from "scratch". Therefore, for most our readers, the internet, *www.ncbi.nlm.nih.gov* and the PUBMED search is the best place to look for PCRs and protocols for screening foods.

In the past, PCR design was based on gene(s) or DNA sequences obtained from screening plasmid clone (50) or transposon libraries (5, 16) for relevant marker, subcloning and sequencing DNA inserts. This approach took considerable time and resources. Now, in less time, we can sequence the entire genome of a single bacterial species, and spend the remainder of our time at the computer annotating its sequence, searching for signature sequences unique to pathogen X. In 1995, the first organism was completely sequenced (20). Since that time, 91 bacterial genomes of several, important human pathogens have been sequenced, annotated, and published (*www.tigr.org*; accessed 2/16/05), including several foodborne pathogens (10, 12, 35, 52, 59, 66, 69, 74, 82, 92). From comparisons of these bacterial genomes, especially between closely related commensals and pathogens, several regions within the chromosome have been identified that appear to be unique to organism X that is tied to its virulence (7, 62, 93), or metabolism (69). With the growing number of bacterial genomes present in public accessible DNA databases, identification and design of PCR for organism can be done *in silico*, on your desktop computer. A priori, of course is that organism X's genome has been sequenced and accessible to the user. With advances in PCR and *in silico* analyses of bacterial genomes, we can amplify, clone and sequence large regions of the bacterial chromosome to quickly identify target DNA sequences for PCR primer design (89, 90)

Access DNA Databases to Retrieve Sequences or Search for DNA Matches. For the researcher, the most important resource, second only to the library and PUBMED, is the DNA database, GenBank at the National Center for Biotechnology Information, National Institutes of Health, Bethesda, Maryland. This database can be accessed via the internet at the following Website: *www.ncbi.nlm.nih.gov*, go to the ENTREZ selection at the top of the page, and then go to GenBank on the next Web page. One can then search the database of sequences by typing in keywords or combination of words for a specific organism, serovar, or gene(s). Prior to this search, it is important to do

your initial research in the library, so that your GenBank search is refined and specific to pull out select sequences from the millions, probably billions, of data base entries present at this Website. The next step is to access a specific GenBank accession, for this exercise we will examine the *Salmonella enterica* Typhimurium LT2 genome at NCBI, GenBank Accession # NC 003917 (Fig. 1.6A, B) and search the annotated genome for the invasion gene *invA* (24, 25) by using the search function in Netscape Navigator for the word "invA" to find the beginning and end of each gene's open reading frame (ORF) (Fig. 1.6C, D). We write down this information and scroll down to the complete sequence to find and copy these sequences (Fig 1.7A). We can paste this sequence for the time being into MSWORD, MSWORDPERFECT, or WORD Notepad and save this file, giving it the organism/gene name. The first three nucleotides should start with ATG, the start codon or rare start codon GTG, and end with TAA, TAG, or TGA, the stop codons. It should be noted, especially with genome sequences, the gene may be in the opposite orientation on the chromosome, requiring inversion of DNA sequence and transcribing the opposite DNA strand to identify start and stop of our ORF. Many DNA software analysis programs can do this for us. We chose the ORF rather than flanking or intergenic regions, because we expect greater selection pressure and less chance for sequence divergence among strains of organism X than these intergenic regions. This is especially important if we are to identify all members of organism X. Now that we have these sequences, we need to determine, *in silico*, whether these sequences are unique to genus *Salmonella* and specifically, the serovar Typhimurium. This can be determined going to BLAST on *www.ncbi.nlm.nih.gov* Website. Click on BLAST and under Nucleotide, click on "nucleotide-nucleotide BLAST (blastn) (Fig. 1.7B)." This will take you to a new site within NCBI that has a box beside "Search". Paste your sequence into this box, and click on the BLAST button (Fig. 1.7B). On the next page, select under the "Format" section, the box titled "or select from" and chose "Bacteria [ORGN]" and click on the FORMAT button (Fig. 1.7C). Allow the BLAST search time to search the database. The time it takes for the search is dependent on gene and the amount of "traffic" at this Website; it is a very popular site with researchers. The results are returned, outlining how many matches there are to your gene sequence. As of 8 February 2005, there were 222 matches with the closest matches, (>90%) to *S. enterica invA* representing various serovars. Other matches are identified, most notably in homologues, genes with similar function, present in *Escherichia coli*. This is expected, as *invA* is part of the type III secretion system present in many human and plant pathogens (40). More importantly, the BLAST results identify for us region of the *invA* sequence to focus on in our primer design. This database search using BLAST is the same approach one would use in analyzing DNA sequence generated from the sequencing of plasmid clones or PCR amplicons. There are however, no guarantees that your gene or sequence will prove useful as a diagnostic marker for organism X, based solely on this database search. You find only what is available on the database, at the time of your search. It is therefore important experimentally to determine the distribution of your candidate gene or allele among

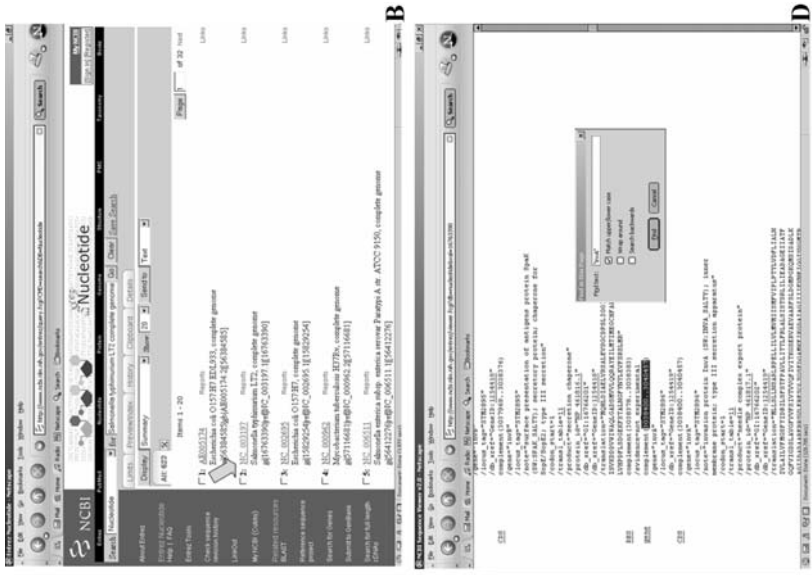

Figure 1.6. National Center for Biotechnology, GenBank DNA database. (A) Entrez. (B) Accessing *Salmonella enterica* Typhimurium LT2 genome GenBank Accession # NC 003917 at NCBI. (C) Search GenBank #NC003917 for "*invA*". (D) Finding the "*invA*" coding sequence (CDS).

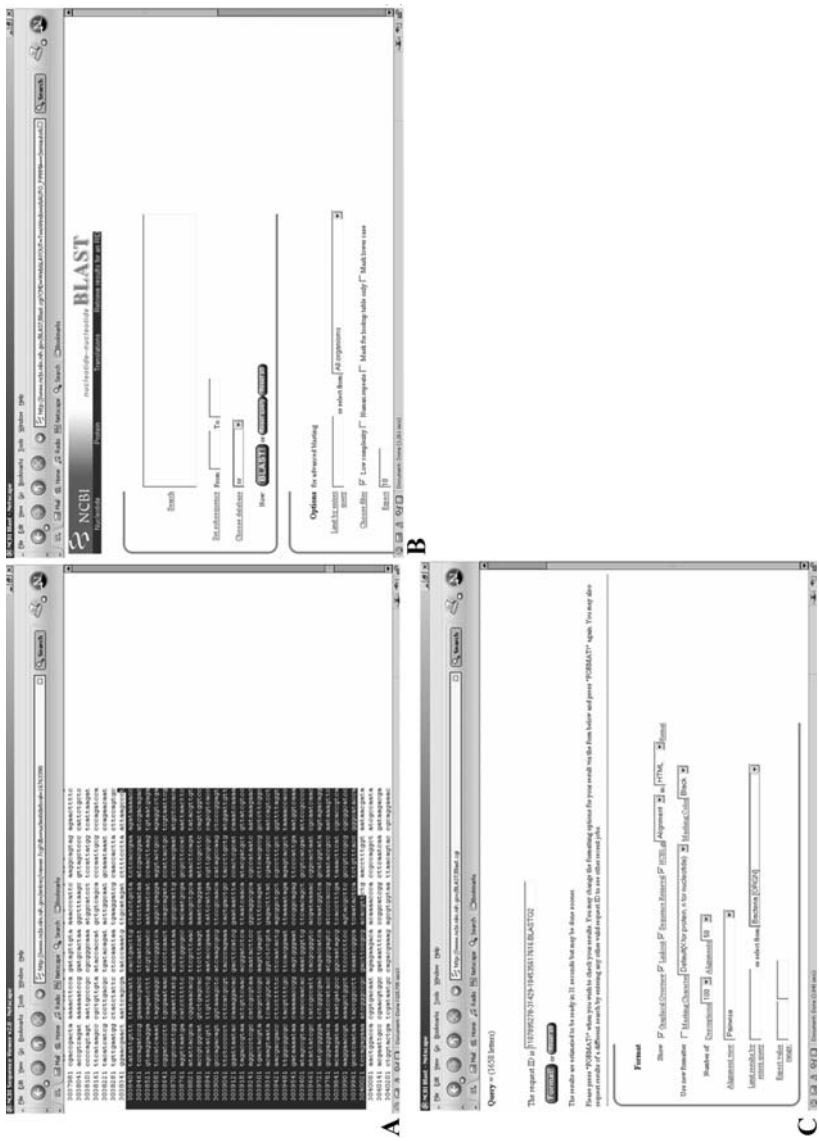

Figure 1.7. National Center for Biotechnology, BLAST Search. (A) Coping "*invA*" coding sequence from GenBank # NC 003917. (B) BLASTn sequence homology search engine. (C) Formatting BLASTn sequence homology search engine.

a sampling of strains, serovars, and closely and distantly related microbes. Despite the presence of *invA* homolog in other genera and species, sequences are divergent enough for this to be a useful genetic marker for detecting *Salmonella* (68, 72).

Now that you have determined *in silico* your candidate genetic marker, you can proceed to analyze your sequence(s) for the best PCR primer pairs. There are several commercially available, as well as Web-based (13; http://dbb.nhri.org.tw/primer/) DNA analysis software packages for designing PCR primers that vary in price, utility, ease, options, or familiarity to the authors. Therefore, we will only provide the reader with general design considerations. First, let us consider in our design the size we want for our amplicon. This consideration is especially important in the development of multiplex PCR where the size of the amplicon identifies the gene or organism present in our sample. Also PCRs sensitivity is influenced by the size of the amplicon. For sensitive, real-time PCR, small amplicons, 75–200 bp are preferred. Next thing to consider is where to concentrate our search for specific PCR primers. From our BLAST search, it appears that the 1st 750 bp of *Salmonella invA* is ideal for our analysis. Also to improve the specificity of our PCR, we need to consider the length of each primer (94). Generally, the minimum default value for many of the PCR primer design algorithms is 18 bp. This value is generated from the probability of finding this exact sequence within the bacterial genome, where for this example; we are dealing with an organism with 50% GC content and 4,000,000 bp genome. The probability of a specific 18 bp sequence is present is $(1/4)^{18} \times 4{,}000{,}000 = 6 \times 10^{-5}$. The smaller the sequence, the greater the likelihood of finding sequence not just once but multiple times within the genome. That is why short 10-mer oligonucleotides have become useful tools for typing bacteria by random amplified polymorphic DNA (RAPD) PCR (51), because based on size and using our calculations we expect to find these sequences at least 4 times within the bacterial genome. Now, having run our analysis, we are presented with all possible primer pairs. Our next step is to select primers for the amplicon size that we want and screen these primer sets further, to identify those that do not form "hairpins" or primer–dimers. We especially want to avoid primers that form hairpins at the 3′ end as this will interfere with the primers annealing precisely to its target sequence and participation of the primer in the DNA extension step in PCR. Primer–dimers and hairpins can affect the specificity and sensitivity of PCR and should be avoided if possible (78). Once the appropriate primer set(s) has been identified, search the GenBank DNA database for match with our primers. With the BLAST search, it is recommended with searches of short sequences to select Bacteria under "or select from" option. This is to limit confusion with random and insignificant matches with the larger animal and plant genomes (10^9 bp) that sometimes occur. Beyond this point, we generally empirically optimize our PCR, using appropriate positive and negative controls, and identifying the magnesium concentration and PCR annealing temperature with the sensitivity and specificity that is best for detecting organism X. We then verify the specificity of our PCR by comparing same strains, serovars, or species against different strains, serovars, and closely and distantly related microorgan-

isms to see if same size PCR amplicon is produced only for those groups of bacteria to which the PCR was intended to identify. Ideally, once our PCR has been optimized, PCR amplicon, of the expected size is only observed among select bacteria that possess the target gene and nothing for all other microorganisms that do not possess this gene. It is at this point too that we verify that our amplicon, with size expected based on the primers designed, is the target gene to which our primers were intended to amplify. We accomplish this by sequencing the PCR amplicon and match resulting sequence against GenBank DNA database using the BLAST algorithm. Our amplicon's sequence should match the *invA* sequences present on the DNA database.

REFERENCES

1. Abdulmawjood, A., M. Bulte, S. Roth, H. Schonenbrucher, N. Cook, M. D'Agostino, M. Burkhard, K. Jordan, S. Pelkonen, and J. Hoorfar. 2004. Toward an international standard for PCR-based detection of foodborne *Escherichia coli* O157: Validation of the PCR-based method in a multicenter interlaboratory trial. J. AOAC Int. **87**:856–860.
2. Al-Khaldi, S.F., K.M. Myers, A. Rasooly, and V. Chizhikov. 2004. Genotyping of *Clostridium perfringens* toxins using multiple oligonucleotide microarray hybridization. Mol. Cell Probes **18**:359–367.
3. Anderson, A.D., V.D. Garrett, J. Sobel, S.S. Monroe, R.L. Fankhauser, K.J. Schwab, J.S. Bresee, P.S. Mead, C. Higgins, J. Campana, R.I. Glass, and Outbreak Investigation Team. 2001. Multistate outbreak of Norwalk-like virus gastroenteritis associated with a common caterer. Am. J. Epidemiol. **154**:1013–1019.
4. Benjamin, M.A., J. Lu, G. Donnelly, P. Dureja, and D.M. McKay. 1998. Changes in murine jejunal morphology evoked by the bacterial superantigen *Staphylococcus aureus* enterotoxin B are mediated by CD4+ T cells. Infect. Immun. **66**:2193–2199.
5. Bilge, S.S., J.C. Vary Jr., S.F. Dowell, and P.I. Tarr. 1996. Role of *Escherichia coli* O157:H7 O side chain in adherence and analysis of an *rfb* locus. Infect. Immun. **64**:4795–4801.
6. Borucki, M.K., S.H. Kim, D.R. Call, S.C. Smole, and F. Pagotto. 2005. Selective discrimination of *Listeria monocytogenes* epidemic strains by a mixed-genome DNA microarray compared to discrimination by pulsed-field gel electrophoresis, ribotyping, and multilocus sequence typing. J. Clin. Microbiol. **42**:5270–5276.
7. Brussow, H., C. Canchaya, and W.D. Hardt. 2004. Phages and the evolution of bacterial pathogens: From genomic rearrangements to lysogenic conversion. Microbiol. Mol. Biol. Rev. **68**:560–602.
8. Buzby, J.C. and T. Roberts. 1997. Economic costs and trade impacts of microbial foodborne illness. World Health. Stat. Q. **50**:57–66.
9. Cha, J.H., M.Y. Chang, J.A. Richardson, and L. Eidels. 2003. Transgenic mice expressing the diphtheria receptor are sensitive to the toxin. Mol. Microbiol. **49**:235–240.
10. Chain, P.S., E. Carniel, F.W. Larimer, J. Lamerdin, P.O. Stoutland, W.M. Regala, A.M. Georgescu, L.M. Vergez, M.L Land, V.L. Motin, R.R. Brubaker, J. Fowler, J. Hinnebusch, M. Marceau, C. Medigue, M. Simonet, V. Chenal-Francisque, B. Souza, D. Dacheux, J.M. Elliott, A. Derbise, L.J. Hauser, and E. Garcia. 2004. Insights into the evolution of *Yersinia pestis* through whole-genome comparison with *Yersinia pseudotuberculosis*. Proc. Natl. Acad. Sci. USA **101**:13826–13831.

11. Chan, K., S. Baker, C.C. Kim, C.S. Detweiler, G. Dougan, and S. Falkow. 2003. Genomic comparison of *Salmonella enterica* serovars and *Salmonella bongori* by use of an *S. enterica* serovar Typhimurium DNA microarray. J. Bacteriol. **185**:553–563.
12. Chen, C., K.-M. Wu, Y.-C. Chang, C.-H. Chang, H.-C. Tsai, T.-L. Liao, Y.-M. Liu, H.-J. Chen, A.B.-T. Shen, J. – C. Li, T.-L. Su, C.-P. Shao, C.-T. Lee, L.-I. Hor, and S.-F. Tsai. 2003. Comparative genome analysis of *Vibrio vulnificus*, a marine pathogen. Gen. Res. **13**:2577–2587.
13. Chen, S.H., C.Y. Lin, C.S. Cho, C.Z. Lo, and C.A. Hsiung. 2003. Primer design assistant (PDA): A Web-based primer design tool. Nucl. Acids Res. **31**:3751–3754.
14. Christensen, J.E., J.A. Stencil, and K.D. Reed. 2003. Rapid identification of bacteria from positive blood cultures by terminal restriction fragment length polymorphism profile analysis of the 16S rRNA gene. J. Clin. Microbiol. **41**:3790–3800.
15. Dahan, S., S. Knutton, R.K. Shaw, V.F. Crepin, G. Dougan, and G. Frankel. 2004. Transcriptome of enterohemorrhagic *Escherichia coli* O157 adhering to eukaryotic plasma membranes. Infect. Immun. **72**:5452–5459.
16. Desmarchelier, P.M., S.S. Bilge, N. Fegan, L. Mills, J.C. Vary, and P.I. Tarr. 1998. A PCR specific for *Escherichia coli* O157 based on the *rfb* locus encoding O157 lipopolysaccharide. J. Clin. Microbiol. **36**:1801–1804.
17. Dimech, W., D.S. Bowden, B. Brestovac, K. Byron, G. James, D. Jardine, T. Sloots, and E.M. Dax. 2004. Validation of assembled nucleic acid-based tests in diagnostic microbiology laboratories. Pathol. **36**:45–50.
18. Donohue-Rolfe, A., I. Kondova, S. Oswald, D. Hutto, and S. Tzipori. 2000. *Escherichia coli* O157:H7 strains that express Shiga toxin (Stx) 2 alone are more neurotropic for gnotobiotic piglets than are isotypes producing only Stx1 or both Stx1 and Stx 2. J. Infect. Dis. **181**:1825–1829.
19. Fischer, S.H., and I. Nachamkin. 1991. Common and variable domains of the flagellin gene, *flaA*, in *Campylobacter jejuni*. Mol. Microbiol. **5**:1151–1158.
20. Fraser, C.M., J.D. Gocayne, O. White, M.D. Adams, R.A. Clayton, R.D. Fleischmann, C.J. Bult, A.R. Kerlavage, G. Sutton, J.M. Kelley, et al. 1995. The minimal gene complement of *Mycoplasma genitalium*. Science **270**:397– 403.
21. Fratamico, P.M., S.K. Sackitey, M. Wiedmann, and M.Y. Deng. 1995. Detection of *Escherichia coli* O157:H7 by multiplex PCR. J. Clin. Microbiol. **33**:2188–2191.
22. Freeman, W.M., D.J. Robertson, and K.E. Vrana. 2000. Fundamentals of DNA hybridization arrays for gene expression analysis. Biotechniques **29**:1042–1055.
23. Fukiya, S., H. Mizoguchi, T. Tobe, and H. Mori. 2004. Extensive genomic diversity in pathogenic *Escherichia coli* and *Shigella* strains revealed by comparative genomic hybridization microarray. J. Bacteriol. **186**:3911–3921.
24. Galan, J.E. and R. Curtiss III. 1989. Cloning and molecular characterization of genes whose products allow *Salmonella typhimurium* to penetrate tissue culture cells. Proc. Natl. Acad. Sci. USA **86**:6383–6387.
25. Galan, J.E. and R. Curtiss III. 1991. Distribution of the *invA*, -*B*, -*C*, and –*D* genes of *Salmonella typhimurium* among other *Salmonella* serovars: *invA* mutants of *Salmonella Typhi* are deficient for entry into mammalian cells. Infect. Immun. **59**:2901–2908.
26. Gannon, V.P., S. D'Souza, T. Graham, R.K. King, K. Rahn, and S. Read. 1997. Use of the flagellar H7 gene as a target in multiplex PCR assays and improved specificity in identification of enterohemorrhagic *Escherichia coli* strains. J. Clin. Microbiol. **35**:656–662.
27. Giesendorf, B.A.J., J.A.M. Vet, S. Tyagi, E.J.M.G. Mensink, F.J.M. Trijbels, and H.J. Blom. 1998. Molecular beacons: A new approach for semiautomated mutation analysis. Clin. Chem. **44**:482–486.

28. Harmon, K.M., G.M. Ransom, and I.V. Wesley. 1997. Differentiation of *Campylobacter jejuni* and *Campylobacter coli* by polymerase chain reaction. Mol. Cell. Probes **11**:195–200.
29. Heid, C.A., J. Stevens, K.J. Livak, and P.M. Williams. 1996. Real time quantitative PCR. Genome Res. **6**:986–994.
30. Herold, S., J. Siebert, A. Huber, and H. Schmidt. 2005. Global expression of prophage genes in *Escherichia coli* O157:H7 strain EDL933 in response to norfloxacin. Antimicrob. Agents Chemother. **49**:931–944.
31. Herrera-Leon, S., J.R. McQuiston, M.A. Usera, P.I. Fields, J. Garaizar, and M.A. Echeita. 2004. Multiplex PCR for distinguishing the most common phase-1 flagellar antigens of *Salmonella spp. J.* Clin. Microbiol. **42**:2581–2586.
32. Higuchi, R., C. Fockler, G. Dollinger, and R. Watson. 1993. Kinetic PCR analysis: Real-time monitoring of DNA amplification reactions. Biotechnol. **11**:1026–1030.
33. Hirsch, H.H. and W. Bossart. 1999. Two-centre study comparing DNA preparation and PCR amplification protocols for herpes simplex virus detection in cerebrospinal fluids of patients with suspected herpes simplex encephalitis. J. Med. Virol. **57**:31–35.
34. Hogberg, A., J.E. Lindberg, T. Leser, and P. Wallgren. 2004. Influence of cereal non-starch polysaccharides on ileo-caecal and rectal microbial populations in growing pigs. Acta. Vet. Scand. **45**:87–98.
35. Holden, M.T., E.J. Feil, J.A. Lindsay, S.J. Peacock, N.P. Day, M.C. Enright, T.J. Foster, C.E. Moore, L. Hurst, R. Atkin, A. Barron, N. Bason, S.D. Bentley, C. Chillingworth, T. Chillingworth, C. Churcher, L. Clark, C. Corton, A. Cronin, J. Doggett, L. Dowd, T. Feltwell, Z. Hance, B. Harris, H. Hauser, S. Holroyd, K. Jagels, K. D. James, N. Lennard, A. Line, R. Mayes, S. Moule, K. Mungall, D. Ormond, M. A. Quail, E. Rabbinowitsch, K. Rutherford, M. Sanders, S. Sharp, M. Simmonds, K. Stevens, S. Whitehead, B.G. Barrell, B.G. Spratt, and J. Parkhill. 2004. Complete genomes of two clinical *Staphylococcus aureus* strains: Evidence for the rapid evolution of virulence and drug resistance. Proc. Natl. Acad. Sci. USA **101**:9786–9791.
36. Holland, P.M., R.D. Abramson, R. Watson, D.H. Gelfand. 1991. Detection of specific polymerase chain reaction product by utilizing the 5′–3′ exonuclease activity of *Thermus aquaticus* DNA polymerase. Proc. Natl. Acad. Sci. USA **88**:7276–7280.
37. Hong, Y., M.E. Berrang, T. Liu, C.L. Hofacre, S. Sanchez, L. Wang, and J.J. Maurer. 2003. Rapid detection of *Campylobacter coli*, *C. jejuni*, and *Salmonella enterica* on poultry carcasses by using PCR-enzyme-linked immunosorbent assay. Appl. Environ. Microbiol. **69**:3492–3499.
38. Hoorfar, J., P. Wolffs, and P. Radstrom. 2004. Diagnostic PCR: Validation and sample preparation are two sides of the same coin. APMIS **112**:808–814.
39. Houf, K., A. Tutenel, L. De Zutter, J. Van Hoof, and P. Vandamme. 2000. Development of a multiplex PCR assay for the simultaneous detection and identification of *Arcobacter butzleri*, *Arcobacter cryaerophilus,* and *Arcobacter skirrowii*. FEMS Microbiol. Lett. **193**:89–94.
40. Hueck, C.J. 1998. Type III protein secretion systems in bacterial pathogens of animal and plants. Microbiol. Mol. Biol. Rev. **62**:379–433.
41. Johnson, W.M., S.D. Tyler, E.P. Ewan, F.E. Ashton, D.R. Pollard, and K.R. Rozee. 1991. Detection of genes for enterotoxins, exfoliative toxins, and toxic shock syndrome toxin 1 in *Staphylococcus aureus* by the polymerase chain reaction. J. Clin. Microbiol. **29**:426–430.
42. Kaplan, C.W., J.C. Astaire, M.E. Sanders, B.S. Reddy, and C.L. Kitts. 2001. 16S ribosomal DNA terminal restriction fragment patter analysis of bacterial communities

in feces of rats fed *Lactobacillus acidophilus* NCFM. Appl. Environ. Microbiol. **67**:1935–1939.
43. Keramas, G., D.D. Bang, M. Lund, M. Madsen, H. Bunkenborg, P. Telleman, and C. B. V. Christensen. 2004. Use of culture, PCR analysis, and DNA microarrays for detection of *Campylobacter jejuni* and *Campylobacter coli* from chicken feces. J. Clin. Microbiol. **42**:3985–3991.
44. Koopmans, M. and E. Duizer. 2004. Foodborne viruses: An emerging problem. Int. J. Food Microbiol. **90**:23–41.
45. Mackay, I.M. 2004. Real-time PCR in the microbiology laboratory. Clin. Microbiol. Infect. **10**:190–212.
46. Marsh, T.L. 1999. Terminal restriction fragment length polymorphism (T-RFLP): An emerging method for characterizing diversity among homologous populations of amplification products. Curr. Opin. Microbiol. **2**:323–327.
47. Marsh, T.L., P. Saxman, J. Cole, and J. Tiedje. 2000. Terminal restriction fragment length polymorphism analysis program, a Web-based research tool for microbial community analysis. Appl. Environ. Microbiol. **66**:3616–3620.
48. Marston, C.K., F. Jamieson, F. Cahoon, G. Lesiak, A. Golaz, M. Reeves, and T. Popovic. 2001. Persistence of a distinct *Corynebacterium diphtheriae* clonal group within two communities in the United States and Canada where diphtheria is endemic. J. Clin. Microbiol. **39**:1586–1590.
49. Maurer, J.J. and M.D. Lee. 2005. *Salmonella*. *In* Mansel Griffiths (ed.), Understanding Pathogen Behavior, Woodhead Publishing Limited, Cambridge, United Kingdom (In Press).
50. Maurer, J.J., D. Schmidt, P. Petrosko, S. Sanchez, L. Bolton, and M.D. Lee. 1999. Development of primers to O-antigen biosynthesis genes for specific detection of *Escherichia coli* O157 by PCR. Appl. Env. Microbiol. **65**:2954–2960.
51. Mazurier, S., A. van de Giessen, K. Heuvelman, and K. Wernars. 1992. RAPD analysis of *Campylobacter* isolates: DNA fingerprinting without the need to purify DNA. Lett. Appl. Microbiol. **14**:260–262.
52. McClelland, M., K.E. Sanderson, J. Spieth, S.W. Clifton, P. Latreille, L. Courtney, S. Porwollik, J. Ali, M. Dante, F. Du, S. Hou, D. Layman, S. Leonard, C. Nguyen, K. Scott, A. Holmes, N. Grewal, E. Mulvaney, E. Ryan, H. Sun, L. Florea, W. Miller, T. Stoneking, M. Nhan, R. Waterston, and R. K. Wilson. 2001. Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. Nature **413**:852–856.
53. Mead, P.S., L. Slutsker, V. Dietz, L.F. McCaig, J.S. Bresee, C. Shapiro, P.M. Griffin, and R.V. Tauxe. 1999. Food-related illness and death in the United States. Emerg. Infect. Dis. **5**:607–625.
54. Moscoso, H., S.G. Thayer, C.L. Hofacre, and S.H. Kleven. 2004. Inactivation, storage, and PCR detection of Mycoplasma on FTA filter paper. Avian Dis. **48**:841–850.
55. Mullah, B., K. Livak, A. Andrus, and P. Kenney. 1998. Efficient synthesis of double dye-labeled oligodeoxyribonucleotide probes and their application in a real time PCR assay. Nucleic Acids Res. **26**:1026–1031.
56. Mullis, K.B., F.A. Faloona, S.J. Scharf, R.K. Saiki, G.T. Horn, and H.A. Erlich. 1986. Specific enzymatic amplification of DNA in vitro: The polymerase chain reaction. Cold Spring Harbor Symp. Quant. Biol. **51**:263–273.
57. Mullis, K.B. 1994. *In* K.B. Mullis, F. Ferré, and R.A. Gibbs (ed.), The Polymerase Chain Reaction. Birkhäuser Boston, Cambridge, MA.
58. Mullis, K.B. 1998. Dancing Naked in the Mind Field. Pantheon Books, New York.
59. Nelson, K.E., D.E Fouts., E.F.Mongodin, J. Ravel, R.T. DeBoy, J.F. Kolonay, D.A. Rasko, S.V. Angiuoli, S.R. Gill, I.T. Paulsen, J. Peterson, O. White, W.C. Nelson, W.

Nierman, M.J. Beanan, L.M. Brinkac, S.C. Daugherty, R.J. Dodson, A.S. Durkin, R. Madupu, D.H. Haft, J. Selengut, S. Van Aken, H. Khouri, N. Fedorova, H. Forberger, B. Tran, S. Kathariou, L.D. Wonderling, G.A. Uhlich, D.O. Bayles, J.B. Luchansky, and C.M. Fraser. 2004. Whole genome comparisons of serotype 4b and 1/2a strains of the foodborne pathogen *Listeria monocytogenes* reveal new insights into the core genome components of this species. Nucl. Acids Res. **32**:2386–2395.

60. Nilsson, W.B. and M.S. Strom. 2002. Detection and identification of bacterial pathogens of fish in kidney tissue using terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA genes. Dis. Aquat. Organ. **48**:175–185.
61. Nordentoft, S., H. Cristensen, and H.C. Wegener. 1997. Evaluation of a fluorescence-labeled oligonucleotide probe targeting 23S rRNA for in situ detection of *Salmonella* serovars in paraffin-embedded tissue sections and their rapid identification in bacterial smears. J. Clin. Microbiol. **35**:2642–2648.
62. Oelschlaeger, T.A. and J. Hacker. 2004. Impact of pathogenicity islands in bacterial diagnostics. APMIS **112**:930–936.
63. Oyofo, B.A., S.A. Thornton, D.H. Burr, T.J. Trust, O.R. Pavlovskis, and P. Guerry. 1992. Specific detection of *Campylobacter jejuni* and *Campylobacter coli* by using polymerase chain reaction. J. Clin. Microbiol. **30**:2613–2619.
64. Panet, A. and H.G. Khorana. 1974. Studies on polynucleotides. The linkage of deoxyribonucleic templates to cellulose and its use in their replication. J. Biol. Chem. **249**:5213–5221.
65. Panicker, G., D.R. Call, M.J. Krug, and A.K. Bej. 2004. Detection of pathogenic *Vibrio spp.* in shellfish by using multiplex PCR and DNA microarrays. Appl. Environ. Microbiol. **70**:7436–7444.
66. Parkhill, J., B.W. Wren, K. Mungall, J.M. Ketley, C. Churcher, D. Basham, T. Chillingworth, R.M. Davies, T. Feltwell, S. Holroyd, K. Jagels, A.V. Karlyshev, S. Moule, M.J. Pallen, C.W. Penn, M.A Quail, M.A. Rajandream, K.M. Rutherford, A.H. van Vliet, S. Whitehead, and B.G. Barrell. 2000. The genome sequence of the foodborne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature **403**:665–668.
67. Paton, A.W. and J.C. Paton. 1998. Detection and characterization of shiga toxigenic *Escherichia coli* by using multiplex PCR assays for stx_1, stx_2, *eae*A, enterohemorrhagic *E. coli hlyA*, rfb_{O111}, and rfb_{O157}. J. Clin. Microbiol. **36**:598–602.
68. Perelle, S., F. Dilasser, B. Malorny, J. Grout, J. Hoorfar, and P. Fach. 2004. Comparison of PCR-ELISA and LightCycler real-time PCR assays for detecting *Salmonella spp.* in milk and meat samples. Mol. Cell Probes **18**:409–420.
69. Perna, N.T., G. Plunkett 3rd, V. Burland, B. Mau, J.D. Glasner, D.J. Rose, G.F. Mayhew, P.S. Evans, J. Gregor, H.A. Kirkpatrick, G. Posfai, J. Hackett, S. Klink, A. Boutin, Y. Shao, L. Miller, E.J. Grotbeck, N.W. Davis, A. Lim, E.T. Dimalanta, K.D. Potamousis, J. Apodaca, T.S. Anantharaman, J. Lin, G. Yen, D.C. Schwartz, R.A. Welch, and F.R. Blattner. 2001. Genome sequence of enterohaemorrhagic *Escherichia coli* O157:H7. Nature **409**:529–533.
70. Porwollik, S., E.F. Boyd, C. Choy, P. Cheng, L. Florea, E. Proctor, and M. McClelland. 2004. Characterization of *Salmonella enterica* subspecies I genovars by use of microarrays. J. Bacteriol. **186**:5883–5898.
71. Prouty, A.M., I.E. Brodsky, S. Falkow, and J.S. Gunn. 2004. Bile-salt-mediated induction of antimicrobial and bile resistance in *Salmonella typhimurium*. Microbiol. **150**:775–783.
72. Rahn, K., S.A. Grandis, R.C. Clarke, S.A. McEwen, J.E. Galan, C. Ginocchio, R. Curtiss III, and C.L. Gyles. 1992. Amplification of *invA* gene sequence of *Salmonella*

typhimurium by polymerase chain reaction as a specific method of detection of *Salmonella*. Mol. Cell Probes **6**:271–279.
73. Rappuoli, R. 2000. Pushing the limits of cellular microbiology: Microarrays to study bacteria-host cell intimate contacts. Proc. Natl. Acad. Sci. USA **97**:13467–13469.
74. Rasko, D.A., J. Ravel, O.A. Okstad, E. Helgason, R.Z. Cer, L. Jiang, K.A. Shores, D.E. Fouts, N.J. Tourasse, S.V. Angiuoli, J. Kolonay, W.C. Nelson, A.B. Kolsto, C.M. Fraser, and T.D. Read. 2004. The genome sequence of *Bacillus cereus* ATCC 10987 reveals metabolic adaptations and a large plasmid related to *Bacillus anthracis* pXO1. Nucl. Acids Res. **32**:977–988.
75. Reeves, M.W., R.J. Arko, F.W. Chandler, and N.B. Bridges. 1986. Affinity purification of staphylococcal toxic shock syndrome toxin 1 and its pathologic effects in rabbits. Infect. Immun. **51**:431–439.
76. Robertson, B.H., F. Averhoff, T.L. Cromeans, X. Han, B. Khoprasert, O.V. Nainan, J. Rosenberg, L. Paikoff, E. DeBess, C.N. Shapiro, and H.S. Margolis. 2000. Genetic relatedness of hepatitis A virus isolated during a community-wide outbreak. J. Med. Virol. **62**:144–150.
77. Rotbart, H.A., M.J. Levin, L.P. Villarreal, S.M. Tracy, B.L. Semler, and E. Wimmer. 1985. Factors affecting the detection of enteroviruses in cerebrospinal fluid with coxsackievirus B3 and poliovirus 1 cDNA probes. J. Clin. Microbiol. **22**:220–224.
78. Rychlik, W. 1995. Priming efficiency in PCR. Biotechn. **18**:84–86, 88–90
79. Saldanha, J., W. Gerlich, N. Lelie, P. Dawson, K. Heermann, A. Heath, and WHO Collaborative Study Group. 2001. An international collaborative study to establish a World Health Organization international standard for hepatitis B virus DNA nucleic acid amplification techniques. Vox Sang. **80**:63–71.
80. Savitt, E.D., M.N. Strzempko, K.K. Vaccaro, W.J. Peros, and C.K. French. Comparison of cultural methods and DNA probe analyses for the detection of *Actinobacillus actinomycetemcomitans*, *Bacteroides gingivalis*, and *Bacteroides intermedius* in subgingival plaque samples. J. Periodontol. **59**:431–438.
81. Schoenhals, G. and C. Whitfield. 1993. Comparative analysis of flagellin sequences from *Escherichia coli* strains possessing serologically distinct flagellar filaments with a shared complex surface pattern. J. Bacteriol. **175**:5395–5402.
82. Shimizu, T., K. Ohtani, H. Hirakawa, K. Ohshima, A. Yamashita, T. Shiba, N. Ogasawara, M. Hattori, S. Kuhara, and H. Hayashi. 2002. Complete genome sequence of *Clostridium perfringens*, an anaerobic flesh-eater. Proc. Natl. Acad. Sci. USA **99**:996–1001.
83. Smith, N.H. and R.K. Selander. 1990. Sequence invariance of the antigenic-coding central region of the phase 1 flagellar filament gene (*fliC*) among strains of *Salmonella typhimurium*. J. Bacteriol. **172**:603–609.
84. Stintzi, A., D. Marlow, K. Palyada, H. Naikare, R. Panciera, L. Whitworth, and C. Clarke. 2005. Use of genome-wide expression profiling and mutagenesis to study the intestinal lifestyle of *Campylobacter jejuni*. Infect. Immun. **73**:1797–1810.
85. Taboada, E.N., R.R. Acedillo, C.D. Carrillo, W.A. Findlay, D.T. Medeiros, O.L. Mykytczuk, M.J. Roberts, C.A. Valencia, J.M. Farber, and J.H. Nash. 2004. Large-scale comparative genomics meta-analysis of *Campylobacter jejuni* isolates reveals low level of genome plasticity. J. Clin. Microbiol. **42**:4566–4576.
86. Thomas, E.J., R.K. King, J. Burchak, and V.P. Gannon. 1991. Sensitive and specific detection of *Listeria monocytogenes* in milk and ground beef with the polymerase chain reaction. Appl. Environ. Microbiol. **57**:2576–2580.
87. Vinje, J., H. Vennema, L. Maunula, C.H. von Bonsdorff, M. Hoehne, E. Schreier, A. Richards, J. Green, D. Brown, S.S. Beard, S.S. Monroe, E. de Bruin, L. Svensson,

and M.P. Koopmans. 2003. International collaborative study to compare reverse transcriptase PCR assays for detection and genotyping of noroviruses. J. Clin. Microbiol. **41**:1423–1433.

88. Vora, G.J., C.E. Meador, D.A. Stenger, and J.D. Andreadis. 2004. Nucleic acid amplification strategies for DNA microarray-based pathogen detection. **70**:3047–3054.
89. Wang, L., W. Qu, and P.R. Reeves. 2001. Sequence analysis of four *Shigella boydii* O-antigen loci: Implication for *Escherichia coli* and *Shigella* relationships. Infect. Immun. **69**:6923–6930.
90. Wang, L., and P.R. Reeves. 1998. Organization of *Escherichia coli* O157 O antigen gene cluster and identification of its specific genes. Infect. Immun. **66**:3545–3551.
91. Watson, J.D., and F.H. Crick. 1953. Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. Nature. **171**:737–738.
92. Wei, J., M.B. Goldberg, V. Burland, M.M. Venkatesan, W. Deng, G. Fournier, G.F. Mayhew, G. Plunkett 3rd, D.J. Rose, A. Darling, B. Mau, N.T. Perna, S.M. Payne, L. J. Runyen-Janecky, S. Zhou, D.C. Schwartz, and F.R. Blattner. 2003. Complete genome sequence and comparative genomics of *Shigella flexneri* serotype 2a strain 2457T. Infect. Immun. **71**:2775–2786.
93. Whittam, T.S., and A.C. Bumbaugh. 2002. Inferences from whole-genome sequences of bacterial pathogens. Curr. Opin. Genet. Dev. **12**:719–725.
94. Wu, D.Y., L. Ugozzoli, B.K. Pal, J. Qian, and R.B. Wallace. 1991. The effect of temperature and oligonucleotide primer length on the specificity and efficiency of amplification by the polymerase chain reaction. DNA Cell. Biol. **10**:233–238
95. Yoo, H.S., S.U. Lee, K.Y. Park, and Y.H. Park. 1997. Molecular typing and epidemiological survey of prevalence of *Clostridium perfringens* types by multiplex PCR. J. Clin. Microbiol. **35**:228–232.
96. Zhang, C., M. Zhang, J. Ju, J. Nietfeldt, J. Wise, P.M. Terry, M. Olson, S.D. Kachman, M. Wiedmann, M. Samadpour, and A. K. Benson. 2003. Genome diversification in phylogenetic lineages I and II of *Listeria monocytogenes*: Identification of segments unique to lineage II populations. J. Bacteriol. **185**:5573–5584.
97. Zhang, X., A.D. McDaniel, L.E. Wolf, G.T. Keusch, M.K. Waldor, and D.W. Acheson. 2000. Quinolone antibiotics induce Shiga toxin-encoding bacteriophages, toxin production, and death in mice. J. Infect. Dis. **181**:664–670.