CHAPTER 2

The Mythology of PCR: A Warning to the Wise

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INTRODUCTION

Most diagnostic PCR tests are a qualitative yes or no, presence or absence of pathogen X. We know what it means if our sample is positive by PCR, reporting back presumptive positive for organism X and a negative PCR result was the end-point for that sample. Were these assumptions correct? The decisions we make based on these PCR results require that we know how to interpret these results and like any other diagnostic test, know its limitations with regards to sensitivity and specificity. Even if your laboratory is only interested in adapting existing PCR methods for identification of pathogens in foods, it is important that you know what the results mean, and know how to recognize and troubleshoot problems as they occur. You can safe guard or at least be prepared to recognize these problems, as they appear, by implementing standard operating procedures and including controls recommended by authors in the chapters discussed in this book. In this section, I will specifically delve into interpretation and understanding of PCR results as well as discuss the limitations, problems, and erroneous assumptions associated with PCR and other PCR based technologies (e.g., real-time PCR).

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INTERPRETATION

Conventional PCR. A sample is positive, by PCR, if an amplicon is produced with the size expected for the primers used. What if the sample yields an amplicon larger or smaller than the size expected for our PCR primers? Is this sample considered positive by PCR? NO!!! This result is referred to in PCR parlance as a nonspecific amplicon, it is ignored, AND *if we do not observe an amplicon with a size expected for primers used, the sample is considered PCR negative*. It is therefore a requirement to always include DNA molecular weight standards, in the appropriate size range for accurately assessing the amplicon's size, and the percentage of agaraose and electrophoresis time needed to adequately separate the molecular weight standards. One needs to also consider other parameters (electrophoresis buffer, buffer strength, voltage, etc.) that affect uniformity of DNA separation across the entire width and length of the agarose gel. For wide gels with many wells or lanes (>10), one may consider placement of the DNA standards in the middle and the outermost wells. With appropriate gel documentation software, the user can, using these well placed molecular weight standards, correct for electrophoresis migration anomalies that produce "smiles" at high voltages. Avoiding electrophoresis at high voltages or circulate/cool the buffer during electrophoresis can prevent this electrophoretic anomaly. With every PCR run, ALWAYS include a positive control so that you can match your sample with the control, and allow adequate separation of your DNA standards, samples, and control so that you do not erroneously report a sample with a nonspecific amplicon as positive. If molecular biology is new to your laboratory, it is advisable to purchase a general molecular biology manual, that details the specifics of gel electrophoresis, includes theory and helps trouble shoot problems commonly associated with the molecular technique (1, 62).

For the experienced molecular biologist, this is rather obvious, but for others, especially the novice, it is easy to be lulled into believing the presence of any PCR product, regardless of size, on the gel means the sample must be positive for organism X. Most genes targeted by PCR have been selected based on their conservation and uniformity within a species, subspecies, serovar or pathotype. These genes are uniform in size. There are, however, exceptions, genes or DNA segments containing repetitive elements or extragenic sequences, the number, size, or presence of which varies within the bacterial population (10, 16, 22, 38, 57, 70). PCRs have been developed to exploit these genetically variable regions for the purpose of genus/species identification (10, 16, 24, 35, 57) and strain typing (25, 56, 57). Here the different size amplicon identifies the genus or species and/or distinguishes strain types. However, a requirement for using any of these PCRs is first the isolation of the organism. For PCR screens of foods, it is advisable to avoid those PCRs that produce, as designed, these variable size amplicons. Unless, an internal probe is included in the PCR screens, for specificity, the technician may confuse a true, nonspecific amplicon in a sample as a positive and erroneously report the sample as such.

Real-Time PCR. Results generated by real-time PCR are generally more straightforward to interpret for a simple question like: is the organism present

in our sample? Rather than visualize the amplicon following PCR, we monitor the increase in fluorescence over time as newly synthesized, amplicon binds to SYBR Green® or the chemically quenched, fluorescent dye is liberated as the amplicon displaces an internally bound, dye-labeled probe. Fig. 2.1 illustrates kinetics of real-time PCR. Note the points on the x-axis, "threshold cycle" (C_T) where the log-linear phase of fluorescence begins for the different target DNA concentrations (43). There is a linear correlation between C_T and DNA concentration, making the PCR quantitative. A sample is considered positive provided it falls within the range of C_T values, demarcated by the PCR's limit of detection, and the background fluorescence associated with the negative or no DNA controls. While real-time PCR surpasses conventional PCR in speed and sensitivity, nonspecific amplicons can result in our erroneously reporting a positive result. SYBR Green® binds to double stranded DNA, regardless of whether it is the expected amplicon, nonspecific amplicon, or primer-dimers. Gradient thermocyclers have become a useful tool in rapidly identifying annealing temperature best for PCR amplification of the target gene while avoiding primer-dimers. We can distinguish nonspecific amplicon(s) from a true positive based on their distinctive DNA melting curves (Fig. 2.2) (59).

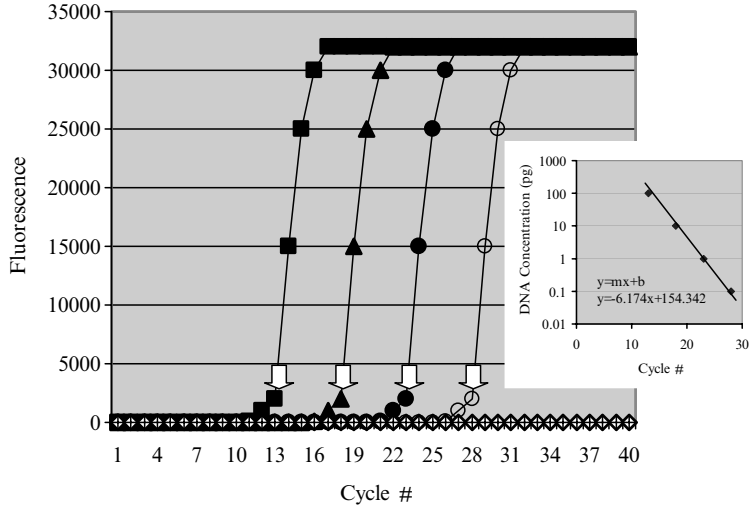

Figure 2.1. Detection of foodborne pathogen X in foods by real time PCR. As amplicon is synthesized, the thermocycler continuously measures fluorescences with each cycle. The PCR product fluoresces due to binding of fluorescent dye, SYBER Green to the double stranded DNA, amplicon as it is formed. When the PCR amplicons are first detected during real time, is a function of the target DNA concentration: ■ (100 pg), ▲ (10 pg), ● (1 pg), (0.1 pg), ◇ (0.01 pg), and + NO DNA control. Arrows identify the "threshold cycle," C_T on the x-axis, # PCR cycles where the log-linear phase of fluorescence begins. The cycle numbers the target DNA concentration was plotted relative to C_T and as shown in the inset, there is a negative, linear correlation between DNA concentration and C_T.

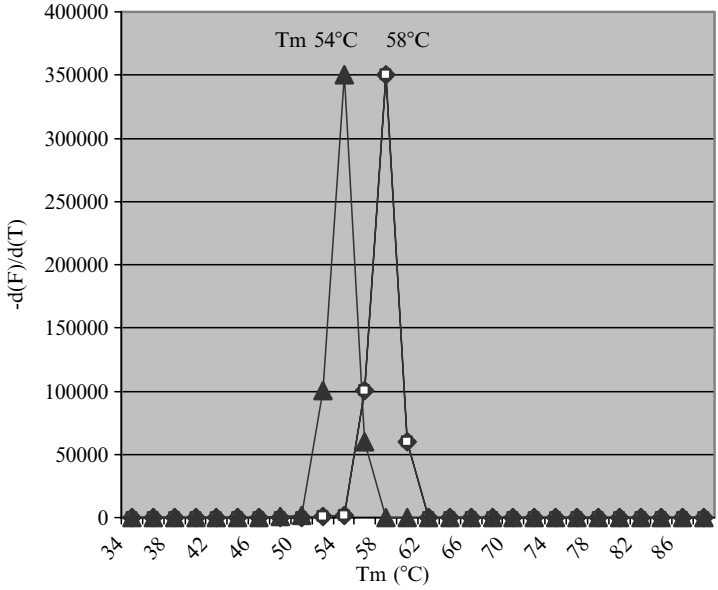

Figure 2.2. Identifying nonspecific PCR amplicons in real time PCR. We can distinguish nonspecific from specific amplicons by measuring the melting temperature (Tm) for each amplicon following real-time PCR. The melting temperature is a reflection of the amplicons's nucleotide sequence, therefore one looks to see if the DNA melting curve for the putative, PCR-positive sample (□) overlaps with that of the positive control (◆) or produces a different melting curve (▲), that is indicative of a nonspecific amplicon.

When we do not observe, directly or indirectly, any PCR product or amplicon of the expected size, the finding is reported as negative. *What does a negative result mean?* For a pure culture, it means our isolate does not possess the gene or gene allele to which our PCR was designed to detect. PCR has become an important diagnostic tool not only in identifying medically important genera (40, 58), but it has been used to identify an organism to species (9, 19, 23, 40), or serotype level (6, 21, 26, 42, 50, 66) as well as determine its antibiotic resistance (20, 27) or virulence potential (2, 55). Depending on the organism and gene(s) or gene alleles associated with resistance to drug X, PCR negative result may indicate: (1) the organism is susceptible to the antibiotic in question (e.g., mycobacterium and isonazid resistance; 27); or (2) PCR negative only means the organism does not possess this gene (e.g.,enterococci and streptogramin resistance; 69) and susceptibility cannot be inferred. Gene screens to assess, genotypically, drug resistance is challenging due to multiple genes and gene alleles associated with resistance to certain antibiotics (8, 15, 64). With regards to detection of multi-drug resistant (MDR) pathogens, while it is

tempting to select antibiotic resistance genes associated with the MDR as probes in PCR screens (31), mobile genetic elements have disseminated these drug resistance genes to innocuous commensals also contaminating foods (32, 63, 74), providing potential for false positives. Gene screens for these MDR loci should therefore be limited to the cultured pathogen. For detection of pathogens in foods, it is imperative that we select a target gene or sequence that is unique to pathogen X, uniform in its distribution within this bacterial population and genetically stable.

If this target gene is strongly associated with genus, species or serotype, a negative PCR translates to this isolate is NOT the species, strain, or serotype identified by this PCR. However, if we apply this very same PCR to screen for the presence of the organism that the PCR was designed to identify, does a negative result mean it is NOT present? We now are confronted with several questions relating to our PCR test's sensitivity and specificity (28, 39), important in assessing, validating and finally standardizing our PCR for screening pathogens in foods (30).

VALIDATION

In optimizing any PCR, we strive to design, identify and develop the primer set(s) for discerning the one genus, species, or strain from multitude of microbial species while being able to detect the fewest cells possible. This is the molecular biology definition of specificity and sensitivity, respectively. To determine specificity, we test our PCR against, many different bacterial strains, closely or distantly related species and/or genera. A PCR specific for *Salmonella*, for example, will produce positive results, amplicon of the expected size, for ALL *Salmonella* species, strains, and serovars but will prove negative for all other bacterial species, especially closely related species (28, 58). If we continue using *Salmonella* as our example, sensitivity is measured by lowest *Salmonella* cell density detectable by our PCR (28, 39). In its infancy, PCR's specificity and sensitivity were determined using pure cultures and at best a food product was spiked with the offending organism and PCR was performed to detect the organism in the processed sample. Only recently have investigators vigorously put PCR through its paces in the real world to validate its utility for rapid detection of pathogens in foods (30).

Validation of any diagnostic PCR involves comparison against another test, considered the "gold standard" for detection. For food microbiologists, the "gold standard" is the bacteriological approach of culture, isolation, and the biochemical or serological confirmatory tests. From this comparison, we determine statistical s*p*ecificity (false-*p*ositives) and se*n*sitivity (false-*n*egatives) of our PCR test (28, 39). A false-positive is when the sample is PCR positive but culture negative, while a false-negative is vice-versa: PCR negative, culture positive. *What is responsible for reporting false-positives and false-negatives and what can we do to minimize this in our food microbiology lab?*

PROBLEMS AND THEIR SOLUTIONS

False positives can be attributed to several things, most you cannot control, but at least one you can: PCR, template, or sample contamination. As discussed in Chapter 4, "Making PCR a Normal Routine of the Food Microbiology Lab," preventative measures and standard operating procedures are essential to avoid these contamination issues. These measures include physically separating DNA and PCR preparation areas from each other as well as from the area where gel-electrophoresis is performed; use of barrier tips, disposable gloves; and cleaning the PCR preparation area with bleach and/or overnight, ultraviolet illumination. As mentioned earlier, PCR amplifies target gene 10^9-fold, producing more than enough molecules per pg-fg of template to serve as template in the next PCR reaction. Following a PCR run and upon opening the tube, we create an aerosol of amplicons that can quickly contaminate our hands, pipettes, and the immediate bench area. Something as simple as disposing of our gloves following the loading of our PCR sample in the agarose gel and before we set up our next PCR reaction, can avoid future PCR "carry-over" contamination. PCR contamination results in considerable down time for the diagnostic laboratory due to the time it takes to identify the source of contamination, and subsequent decontamination of the affected area or disposal of the contaminated reagent(s). Alternatively, some labs substitute thymidine with uracil in the PCR reaction mix and subsequent pretreatment of all PCR reaction mixes with uracil DNA glycosylase prior to running these reactions in the thermocycler (41). The principle behind this method is that during PCR, amplicons incorporate uracil; the amplicon is now susceptible to hydrolysis by uracil DNA glycosylase, and eliminated prior to each subsequent PCR run. Therefore, erroneous reporting of false-positives due to PCR contamination is eliminated.

As synthesis of the amplicons is identified in "real-time" with newer, PCR fluorescence-based detection technologies, tubes never have to be opened following the initial PCR reaction set-up. With conventional PCR, we can identify PCR contamination when negative or NO DNA controls turn positive. For an experienced lab, something is amiss, when the number of PCR positives greatly exceeds frequency the lab normally encounters for this PCR test or incidence reported in the literature AND subsequent culture results do not correlate with the PCR (i.e., increase in false positives). This can be observed with real-time PCR as lower C_T than encountered for past PCR-positive samples, indicative of high-cell density or template/target concentration, and fails to yield the organism upon culture. As PCR can be extremely sensitive, great care must be taken in sample preparation to avoid cross-contamination. Inclusion of a negative control, sample prep with every PCR run will be useful in identifying cross-contamination, as a positive PCR for this negative control would definitely be indicative of template/sample contamination. Anytime when its evident there is PCR contamination, discard the results for that PCR run, discontinue any future PCRs, and identify and correct the problem immediately before rerunning PCR on any past or future samples.

Nonspecific PCR amplicons can also result in erroneously reporting a sample positive for pathogen X. This can be especially problematic for real-time

PCR using SYBR Green® to detect PCR products and multiplex PCR: the multiple gene screens, single PCR test where the size of the amplicon identifies: genus, species, serotype, or strain. For real-time PCR, we can identify this problem by measuring the amplicon's Tm from the DNA melting peak as stated earlier or run sample PCR on gel side-by-side with positive control to see any differences between the two in their size. Tweaking the PCR conditions to improve its stringency can sometimes eliminate these nonspecific amplicons. This can be done by empirically identify annealing temperature or $MgCl_2$ concentration, that eliminates signal for our "false-positive" sample while not affecting the positive control. To increase the stringency of the PCR, one increases the annealing temperatures and/or lower the $MgCl_2$ concentrations in the reaction mix.

Another way we can improve the specificity of our PCR and reporting false positives, is to use PCR that incorporates internal: nested PCR primers (39); DNA: DNA hybridization or capture probes (28, 45); molecular beacon (37); or TaqMan probe (29). These PCRs have improved specificity because the internal capture or detection probes can distinguish between the real amplicon and nonspecific amplicons, by binding to the complementary sequence within the target amplicon during PCR or at DNA: DNA hybridization step. These internal probes also heighten the sensitivity of the PCR at least 100-fold (28, 45).

False-Positives and Dead vs. Live Bacterial Cell Debate. Even when PCR is running optimally, there may not always be 100% agreement between PCR and culture. The reasons for false-positives are not completely understood. Several explanations have been offered and include: (1) the bacteria are in a viable, nonculturable state (61); (2) injury of the bacterial cells during food processing (52); or (3) the bacteria are dead (48). One can obtain variable culture results alone depending on: (1) whether to include a step(s) that allows for the recovery of injured cells (13, 33); (2) the type of enrichment broth (18) and culture conditions (12) used, or (3) the use of a delayed, secondary enrichment (72, 73) and may explain the disconnect sometimes observed between PCR and culture results.

Depending on where samples are taken within the continuum of food processing steps, especially at Critical Control Point (CCP) designed to reduce or eliminate the pathogen, (e.g., heating), PCR may not be able to distinguish live, dead or damaged cells. In fact, we routinely boil bacteria or wash cells in ethanol to prepare template for PCR, conditions that readily and rapidly kill bacteria. Therefore, one may consider where and when to use PCR in assessing product contamination with pathogen X. For a process that readily ruptures or dissolves the bacterial cell, pre-DNAse treatment step can remove residual DNA carried over from dead, lysed cells (51). However, a significant proportion of heat-treated cells remain intact, dead, and suitable as template for PCR (51). We still need to know whether CCP was effective at eliminating the pathogen or reducing it to an acceptably safe level. PCR still affords us the opportunity to identify the few cells still viable following CCP step, (e.g., pasteurization), by using RNA as the template. Unlike DNA, RNA has short-half life in the bacteria cell (34), as genes are turned on and off as the cell grows and responds to its environment.

Upon cell death, these mRNA transcripts are quickly degraded. There has been considerable interest in using RNA as the template for diagnostic PCR to detect the few viable cells remaining in the sample (17, 46, 52, 65, 75). This can be accomplished by converting RNA to its complementary (c) DNA copy with the retroviral reverse transcriptase, at which point the cDNA can serve as template in standard PCR. This procedure is referred to as reverse-transcriptase PCR. The challenge currently is identifying a constitutively, expressed gene that has sequence unique to the organism and has a short, mRNA half-life, especially upon death of the bacterial cell (75). RNA turnover in the bacterial cell is dependent on its intracellular ribonucleases, and like any enzyme once denatured it becomes inactive and the RNA therefore persists, which may explain the long half-life of RNAs following thermal inactivation of the bacterial cell (47). Therefore, there are times when culture continues to be necessary in assessing microbial risk following food processing step at CCP and other instances where PCR trumps culture in the detection of foodborne pathogens (see below).

Finally, we are left to consider *v*iable *b*ut *n*on*c*ulturable (VBNC) bacteria and PCR. We know bacteria can enter a physiological state where, with the microscope, we know they are present and viable, as determined using viability stains, but we are unable to plate them from sample X. This VBNC state may result from cellular injury (14), adaptation to a harsh, oxygen-poor or nutrient deplete environment (5, 7, 71) or subsequent transformation from planktonic to sessile state in biofilms (11). In foods, the VBNC state may be the consequence of cellular injury/damage and may require a recovery period, in a preenrichment broth, before the cells can be cultivated. Organisms like *Vibrio* and *Campylobacter* can readily enter VBNC state, especially in aquatic environments (54, 60). Although regarded as a foodborne pathogen, *Campylobacter* is also recognized as the cause of several waterborne outbreaks in the United States (4, 36). With *Campylobacter*, the VBNC state may be due to physical or chemical agent that damages the cell, or nutrient depletion or limitation triggers a physiological change to a survival state. When *Campylobacter* enters the VBNC state, its cell morphology changes from helical to coccoidal. Pathogens can revert back from this nongrowing, VBNC state into actively growing; cultivatable state, under the right conditions in vitro (7, 71) and cause disease in its animal host (53). It may be that we are unable to detect it in this state using our current selective, enrichment media because of the antibiotics in the media that interfere with cellular repair and changes to the cell wall necessary to resume growth (67, 68). Where our culture-based approaches currently have failed, PCR offers the opportunity for the pathogen's detection, especially in its VBNC state (3, 49, 52).

PCR Inhibitors, Limits of Detection, and False-Negatives. False-negatives, PCR-negative, culture positive samples are attributed to two major factors: PCR inhibitors or the PCR's limit of detection. PCR inhibitors may be attributed to the food sample itself or the enrichment used to amplify the target organism. We can often remove these inhibitors by using simple DNA affinity, spin columns to produce clean DNA template for PCR, making samples generally recalcitrant to PCR (e.g., soil) pliable for PCR-based screens and analyses.

Chapter 4, "Sample Preparation for PCR" will go into more detail concerning sample preparation and preparation of template that is free of PCR inhibitors. More recently, diagnostic PCRs for screening foods have been adapted to include an "internal control" in the sample screened in order to eliminate possibility of extraneous factors (e.g., PCR inhibitors) from factoring into interpretation of PCR negative results. "Internal amplification control" is the cloned, positive-control amplicon where an internal region has been removed (44). As template in PCR, "internal amplification control" produces a smaller sized-amplicon. The plasmid DNA bearing our "internal amplification control" is included with sample template in PCR. If the sample is negative for organism X, a single amplicon, corresponding in size to that expected for the "internal amplification control." However, for a positive sample, two amplicons are produced; one that corresponds in size to that expected from amplification of the organism X's targeted gene and the other corresponds in size to that expected for the internal control.

For most PCR beginners, false-negatives due to PCR's insensitivity to detect a single-cell per sample appear to be a paradoxical, if not a heretical statement. You have probably read many research papers and believe their claim that their PCR can detect a single cell/ml of a sample. Is this really possible? With PCR, we are generally dealing with reaction mix volumes that range between 10 and 100 µl to which we may add 1 or 10 µl of the sample, once its been processed for PCR. What is the probability that you detect 1cell/ml by PCR, if you were to take 0.001 ml or 1 µl, once from that sample? Knowing Poisson distribution, we know that odds are very small that we can detect it. However, if we took multiple aliquots from this same sample, a most-probable number approach, we would improve our chances of detecting this organism by PCR. The reality is that for most PCRs the limit of detection is 1–1000 cells per 1 µl sample template run, which translates to 1,000–1×10^6 cells/ml. Therefore, if we relied on PCR alone, and discounting PCR inhibitors, does a PCR negative sample mean the organism is NOT present? Ideally, one wants to use the PCR that is the most sensitive for identifying pathogen X in our food product. How might we improve our chances of detecting our pathogen knowing these limitations and assuming the organism might be present in our specimens at levels <1000 cells/ml? One approach is to concentrate cells into a smaller volume, or include an enrichment step that amplifies what few cells are present to levels above the PCR's threshold for detection (Chapter 3). For the latter, short enrichment period may be sufficient to bring cell density of the pathogen above the detection limits of the PCR. Enrichments have been especially adapted to PCR protocols for foods due to the necessity of processing the large sample volumes associated with screening foods from pathogens.

CONCLUSIONS

One must be aware of the limitation of any diagnostic test, and PCR is no exception. Will PCR soon be the substitute for current culture or immunological tests for foodborne pathogens? Probably not for all pathogens, but it will

become standard tool for detecting foodborne protozoans and viruses, pathogens that are currently recalcitrant to culture-based methods of detection. PCR will become an important tool in identification of serotypes and pathotypes. It can be a useful part of any detection scheme, helping with decisions as to which samples and enrichments to focus our efforts towards (39). Of course, acceptance and implementation of PCR in the diagnostic laboratory requires an understanding of its mechanics, meaning of results, the test's limitations, and being able to recognize problems and trouble-shoot them as they arise.

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