WHAT WILL PCR BRING TO SHRIMP FARMING: CONTRIBUTION, COMPROMISE OR CONFLICT?

SU CHEN[1], MUDJEKEEWIS D. SANTOS[2] AND JEFF A. COWLEY[3]
[1]GeneReach Biotechnology Corp, Chief Operating Officer. No. 19, Keyuan 2nd Rd, Central Taiwan Science Park, Taichung 407, Taiwan
E-mail: suchen@genereachbiotech.com
[2]National Fisheries Research and Development Institute, 940 Quezon Ave., Quezon City, Philippines
E-mail: mudjiesantos@yahoo.com
[3]CSIRO Food Futures National Flagship, CSIRO Livestock Industries, Queensland Bioscience Precinct, St. Lucia, QLD 4067, Australia.
E-mail: Jeff.Cowley@csiro.au

Importance of pathogen diagnosis for managing shrimp health

Health management based on capabilities to rapidly and accurately detect pathogens and strategies to prevent and treat disease have been pivotal to the success of livestock industries, particularly those involving species farmed intensively such as chickens and pigs. With such terrestrial species, health management systems incorporating vaccines and other disease prevention measures are relatively well established. However, with aquatic species, the rapid expansion of intensive aquaculture industries in the past few decades has resulted in challenges to overcome regular onslaughts of newly emerged diseases. In the context of marine shrimp culture, devastating viral diseases, in particular, have proved difficult to combat due to the lack of effective vaccines. For this reason, sensitive virus detection methods and/or the use of virus-free seedstock to exclude infections from entering ponds are the current best practices for controlling disease in cultured shrimp.

To address massive reductions in production output of farmed shrimp that occurred worldwide during the 1990's due to viral disease, domesticated specific pathogen free (SPF) breeding populations of the Pacific white shrimp (*Penaeus vannamei*) were successfully established at the Oceanic Institute in Hawaii (Wyban *et al.* 1992). The commercial availability of these SPF *P. vannamei* in the late 1990's resulted in their extensive uptake by shrimp aquaculture industries in both Southeast Asia and the Americas to circumvent production losses. Due to the massively improved farm production yields that resulted from the uptake of SPF *L. vannamei*, substantial efforts are currently underway to domesticate the giant tiger shrimp (*Penaeus monodon*) and select for SPF breeding stocks to replicate and further improve the commercial successes achieved with SPF *P. vannamei* (Withyachumnarnkul *et al.* 1998. Preston *et al.* 2009, Preston and Coman 2009). Even with improved farm yields through farming SPF *P. vannamei*, the global demand for shrimp product still outstrips supply and thus shrimp will remain a relatively high priced luxury food item until production can be enhanced further, possibly through the use of SPF *P. monodon* selected genetically for fast growth (Preston *et al.* 2009, Preston and Coman 2009).

Robust bio-security measures, including the screening of broodstock and/or seedstock for potential viral pathogens, are essential for preventing their entry into farm ponds by virus transmitted either vertically or horizontally to seedstock. Therefore, the application of rapid, sensitive and high-throughput diagnostic methods to select for high health and virus-free seedstock provides a means by which disease risks can be reduced and production yields enhanced. Such diagnostic methods are also pivotal to early pathogen detection in farm ponds to allow harvesting before disease outbreaks occur and for quarantine purposes to prevent disease spreading to neighboring ponds. Moreover, when tests can be refined to distinguish between virus strains or genotypes, they can provide an epidemiological tool to trace its origin or track its spread during disease outbreaks.

Sensitive and accurate pathogen detection methodologies are critical for the implementation of effective disease treatment or prevention measures. In addition to sensitivity and specificity, an ability to quantify pathogen infection loads can be crucial to determining inherent disease threats. For example, in the use of polymerase chain reaction (PCR) to detect infectious hypodermal and hematopoietic necrosis virus (IHHNV), false positives can occur in *P. monodon* that contain a non-infectious and chromosomally-integrated IHHNV genome of similar sequence (Tang and Lightner 2006). Once the reason for these false positives became understood, they could be avoided by designing a PCR test that employed primers with sequence mismatches to the integrated IHHNV genome sequence (Kwok *et al*. 1990, Tang *et al.* 2007). Another challenge for accurate pathogen diagnosis using PCR is that it is susceptible to false negatives resulting from genome sequence variation in the pathogen being detected. For example, the yellow head virus (YHV) complex is currently know to comprise at least 6 distinct genotypic variants (Cowley *et al*. 1999, Wijegoonawardane *et al*. 2008a), of which only two have been identified to be virulent and capable of causing disease. As some PCR tests only detect some genotypes (Wijegoonawardane *et al*. 2008b), care is needed in interpreting PCR data. Attempts have been made to assist diagnosis of YHV variants through the design of a group-specific PCR test capable of detecting all genotypes for use in conjunction with tests providing typing capabilities (Cowley *et al*. 2004, Wijegoonawardane *et al*. 2008b, 2010). Importantly also, as locations that require shrimp pathogen diagnosis can be somewhat remote, as hatchery durations are relatively short, and as disease and mortalities can occur rapidly, diagnostic methods that provide a fast data turn-around time are beneficial (Teng *et al*. 2006).

To help confirm the outcomes of remedial measures and treatments to combat pathogens, it is important that any diagnostic system not only be very sensitive, but also possess an ability to accurately quantify infection severity. In contrast to viral pathogens, accurate diagnosis of bacterial infections involving vibrio spp. and necrotizing hepatopanceatitis (NHP), for example, can be instructive on appropriate drug types and doses needed for effective treatment (Bell and Frelier 1991). Even in the absence of effective treatment methods for shrimp viruses, strategies can be implemented to minimize the risk of disease escalation and spread based on the nature of the virus, the species of shrimp cultured, the severity and prevalence of infection as well as the water quality and culture intensity. Moreover, flowing from accurate viral diagnoses, deficiencies in bio-security systems can be identified, measures can be taken to safeguard neighboring ponds and surveillance policies can be implemented as early warning systems to circumvent disease-induced production losses.

Table 1. Three primary factors in which pathogen diagnosis can aid in the health management of cultured shrimp

Factor	*Role of diagnosis*	*Criteria*
Prevention	Confirmation of seedstock quality Evaluation of culture environment Epidemic prevention at early stage Tracking of pathogens	Sensitivity Specificity Typing
Detection	Pathogen confirmation Inference of infectious cause	Specificity Quantitative ability Strain typing
Treatment	Monitoring of treatment outcomes Optimal follow-up procedures	Quantitative ability Sensitivity Strain typing

In summary, due to the unavailability of effective medications/vaccines or of sufficient SPF broodstock to supply the worlds shrimp culture industries with seedstock in combination with inherent difficulties in maintaining farm-level bio-security due to increasing farm densities, pathogen diagnostic methods such as PCR remain pivotal to the health management, expansion and sustainability of these industries.

Use of PCR for shrimp virus diagnosis

Since its invention in 1985 (Mullis *et al*. 1986), various PCR-based DNA amplification methodologies have become widely adopted as both molecular research tools and as diagnostic tests. As PCR can detect DNA with extremely high sensitivity and specificity, is relatively simple to perform, is amenable to high-throughput application and is capable of accurately quantifying nucleic acid copy numbers through the use of various fluorescence detection systems, it has become the method of choice for shrimp virus detection. As listed in Table 2, the Office International des Epizooties (OIE) recommends the use of several PCR methods to detect shrimp viruses of global concern. The only commercial shrimp virus diagnostic kit currently endorsed by the OIE also employs a sensitive and semi-quantifiable nested PCR method (URL1).

As also listed in Table 2, several methods other than PCR remain endorsed by the OIE to diagnosis shrimp viral pathogens and/or disease. For example, light microscopy of wet mount or histological sections can be used to detect occlusion bodies or other cellular pathology characteristic of viral infection and transmission electron microscopy of ultrathin sections can be used to detect virus nucleocapsids or virions that are structurally distinct for the different viruses. Antibody-based viral protein detection methods based on the enzyme-linked immunosorbent assay (ELISA), immuno-dot blots, and rapid colorimetric test strips for pond side use are also endorsed for detecting viral infection. As an alternative to PCR, viral nucleic acid can be detected in histological tissue sections using *in situ* hybridization (ISH) or, following blotting of nucleic acid onto charged nylon membranes, by dot-blot

Table 2. Recommended diagnostic methods for six shrimp viruses summarized from the Manual of Diagnostic Tests for Aquatic Animals 2006, OIE

	WSSV	*TSV*	*IHHNV*	*YHV*	*BPV*	*MBV*
Bioassay	V	V		V		
In situ hybridization	V	V	V	V	V	V
Dot blot			V			V
PCR			V		V	
Nested PCR	V					V
RT-nested PCR				V		
RT-PCR		V		V		
Real-time PCR		V				
Antibody-based methods (immunoblot/ELISA/ immunohistochemistry)		V	V	V	V	V
Microscopy methods	V			V	V	
DNA sequencing	V					
Cell culture/artificial media growth				V		

hybridization using labeled virus-specific RNA/DNA probes (Nunan and Lightner 1997, Phromjai *et al*. 2002). In terms of relative virus detection sensitivity, nested PCR and isothermal DNA amplification approaches are the most efficient, detecting viral DNA/RNA down to very low levels (ie. <10 copies), followed by PCR (<100 copies). Virus detection using ISH, dot blot hybridization, ELISA and protein immunoblot methods has been found generally to be about two orders of magnitude less sensitive, with microscopy and rapid pond-side test strip methods being somewhat less sensitive again.

Due to its simplicity, speed and a pathogen detection sensitivity several orders of magnitude higher than any other methodology, of those available, PCR is used most commonly to diagnose shrimp viruses (Shekhar *et al*. 2006). To expand on the characteristics of PCR that make it the diagnostic method of choice;

A. PCR is now a routine molecular biology technique taught widely at universities. The test is easy and fast to perform, uses widely available reagents and in many cases, ready-to-use reagent premixes, and is highly amenable to simple kit-based commercial implementations. Bioinformatics methods are available to assist in selecting unique PCR primer sequences for use at defined annealing (Tm) and reaction conditions. Moreover, very little, <100 nucleotides (nt), of viral genome sequence information is required for the design of a robust PCR.

B. PCR amplification results in the geometric doubling of the target DNA sequence number at each thermal cycle, thus affording exquisite sensitivity. As PCR primers

are generally about 20 nt in length, with four nucleotide possibilities (A, T, C, G) at each position in the primer, the chance of any two biological sequences matching perfectly with a PCR primer sequence is 1 in 4^{20}, equal to 1 in 1.1 x 10^{12}. Since the human genome is ~3.4 x 10^9 base pairs (bp) long and the largest genome currently known of *Amoeba dubia* is ~6.7 x 10^{11} bp long (Parfrey *et al*. 2008), the potential for a PCR primer 20 nt or longer matching perfectly with two or more independent sequences is improbable. Moreover, as both a forward and reverse PCR primer are usually needed to extend the target DNA in either direction, this adds to its specificity. Whilst this ignores the fact that some DNA sequences will be more similar due to them sharing common evolutionary origins, for example, even if a primer should bind to a closely related sequences, the chances of any two primers binding serendipitously in appropriate orientations at nearby locations to promote DNA amplification is remote (see Section V). Because of this, most diagnostic PCRs will posses good specificity if other general primer design rules are followed with care.

In the logarithmic amplification phase, each cycle of the PCR results in a doubling of the number of DNA copies. Thus, over 20 amplification cycles, the target sequence will be duplicated 2^{20} or around 10^6 times. If an internal region of this DNA product is subsequently amplified in another reaction using separate PCR primers, similar numbers of this nested PCR DNA product can be amplified, thus vastly enhancing the detection sensitivity of a DNA template present in extremely low abundance.

C. In addition to viruses with a DNA genome, viruses with an RNA genome can be readily detected by use of reverse transcription-PCR (RT-PCR) in which viral RNA is first converted to complimentary (c)DNA using reverse transcriptase prior to PCR amplification. Using buffer systems formulated to accommodate efficient cDNA synthesis in addition to *Taq* DNA polymerase PCR amplification, RT-PCR tests can be integrated into a single tube reaction.

D. PCR can be designed to provide either semi-quantitative or accurate quantitative data using several methodologies. Accurate nucleic acid quantification can be obtained by real-time PCR using various fluorescence detection systems directly related to amplified DNA copy numbers and employing a TaqMan probe (Livak *et al*. 1995), a molecular beacon (Ortiz *et al*. 1998) or a Light Cycler probe (Loeffler *et al*. 2000). Whilst there is also an AmpliSensor detection system reliant on PCR primer consumption (Chen *et al*. 2003), most inexpensive and easily implemented real-time PCR systems employ SYBR-Green dye as a non-specific means of detecting amplified double-stranded DNA in combination with melting curve analysis to authenticate the DNA product. In general, the resolution of real-time PCR in quantifying DNA copy numbers is excellent as it can readily discriminate <2-fold differences over a 10^6-fold linear range.

Apart from absolute quantification reliant on the amplification of a dilution series of DNA of know copy number to generate a standard calibration curve (Freeman *et al*. 1999), relative quantification of template amounts can also be obtained by real-

time PCR amplification of an endogenous host DNA, that is stable in copy number across various cell types, using the same sample (Pfaffl 2001). An advantage of relative quantification is that it accommodates variations in tissue type and amount as well as DNA degradation and extraction efficiency that can cause substantial variations in the amount of template added to the reaction. For example, if 95% of the extracted DNA is degraded in a tissue sample, only 5% of the target DNA will be amplified, but correspondingly, only 5% of the endogenous control DNA will also be amplified. Thus the PCR cycle threshold (Ct) ratio of the target and control DNAs will remain constant irrespective of any variations that affect the quality of quantity of DNA added to the reaction.

In addition to real-time PCR, conventional PCR can provide some measure of DNA semi-quantification based either on relative DNA product yields detected after agarose gel electrophoresis or by competitive PCR methods (Rowe *et al*. 1997). In the latter method, the relative amount of template in any sample can be inferred from the relative amounts of DNA products of different sizes detected in a gel. Such conventional and real-time PCR test designs enhance the functionality of methods used for virus diagnosis.

E. The possibility of designing multiplexed primer systems to co-detect different pathogens present within a sample in a single reaction is another reason why PCR offers immense potential. Such multiplexed PCR systems can reduce reagent costs markedly and provide significant advantages when used to detect viruses causing similar symptoms or for producing and maintaining breeding stocks SPF for several viruses where the changes of infection are low. However, adding two or more primer sets for different targets within a single reaction can increase competition for dNTPs and *Taq* DNA polymerase, which can jeopardize PCR detection efficiency when multiple targets are present (Chamberlain *et al*. 1991). Indeed in circumstances where the variation in amounts of two different target DNAs is large, and the amount of one approaches the detection limit of PCR, its amplification can be quenched resulting in a false negative result (Bej *et al*. 1990). In all other circumstances, and as long as primers have been appropriately designed, multiplexed PCR systems detecting two different viruses are likely to succeed (Cowley *et al*., 2004). However, the risks of false negative results due to resource competition will escalate as more sets of primers for different targets are incorporated into a multiplexed PCR. Based on experience, four is the maximum number of targets that can be accommodated within multiplexed PCR protocols for shrimp viruses (Su Chen, unpublished data). Although it is also possible to incorporate quantity-limited primers to amplify an endogenous DNA in a multiplexed PCR, the application of such tests in diagnostic applications requires immense care in test design, validation and in data interpretation.

F. PCR can also be used for genotyping some viral pathogens using various strategies such as amplified fragment length polymorphism (AFLP)-PCR (Mueller and Wolfenbarger 1999), PCR-restriction fragment length polymorphism (RFLP)

(Pourzand and Cerutti 1993), direct sequencing of DNA products amplified by PCR, PCR microarrays and the identification of single nucleotide polymorphism (SNP) (Cargill *et al*. 1999).

In summary, the nature of the PCR method provides numerous advantages for shrimp virus diagnosis including exquisite sensitivity and specificity, speed and adaptability to high throughput systems, ability to accurately quantify infection loads, ability to co-detect or distinguish viral strains/genotypes, and its general acceptance as an effective and robust diagnostic tool. These virtues meet most requirements for pathogen diagnosis and disease prevention and treatment essential for managing the heath of cultured shrimp as summarized in Table 1. With human diseases, PCR has been applied widely for pathogen/disease diagnosis and pathogen genotyping. In support of its adoption, the Food and Drug Administration (FDA) in the USA has endorsed the use of several commercial PCR tests for pathogen detection (Nolte *et al*. 1995) including influenza virus, hepatitis C virus and human immunodeficiency virus. Some concerns, however, persist in the use of PCR, particularly regarding the choice of methodologies to achieve maximum diagnostic accuracy whilst avoiding any test or technical inadequacies with potential for generating either false-positive or false-negative results.

The same concerns about test and/or technical inadequacies resulting in inaccurate data arise when PCR is applied to detect shrimp viruses. There are also some requirements specific to shrimp virus detection not encountered in human disease diagnosis. Whilst PCR testing proficiency and data quality is not regulated as stringently as for pathogen diagnosis in humans, the economic benefits obtainable through appropriate responses to PCR detection of shrimp pathogens can be substantial. In the following three sections, the PCR method and its potential problems (Section III), the specific requirements for sampling, statistics, and epidemiology (Section IV) and the inherent limitations of PCR in diagnosing shrimp pathogens (Section V) will be discussed.

PCR testing requirements for pathogen diagnosis

Through the availability of premixed commercial reagents and test kits, PCR has become a simple and robust diagnostic method. Problems with PCR efficacy relating to pathogen detection efficiency and diagnostic accuracy (avoidance of false-negative or false-positive results), can arise due to many factors. These can include inadequacies in the test design itself and/or its inappropriate application and interpretation. However, problems also commonly arise from inadequacies in sample collection and storage methods and from technical unfamiliarity with extraction requirements to obtain high quality nucleic acid, rather than in performing the PCR *per se*. These technical issues can result in the extracted nucleic acid being too degraded to detect reliably or in the presence of various interference substances that can inhibit PCR, as described in more detail below.

Nucleic acid concentration effects. It is widely recognized that *Taq* DNA polymerase activity can be inhibited in the presence of high concentrations of nucleic acid. In diagnostic applications, it is generally recognized that 50 ng to 200 ng of DNA or cDNA

per 20 ul reaction is well tolerated and that ideally, no more than 1 µg should be used to ameliorate any nucleic acid concentration effects (Dieffenbach and Dveksler 1995). A common error made by less experienced laboratory technicians is to use more tissue for nucleic acid extraction than required, or to resuspend nucleic acid pellets in less buffer than recommended in the false expectation of improving PCR detection sensitivity.

Protein and long-chain polysaccharide inhibitors. Most commercial nucleic acid extraction reagents, such as TRIzol™ reagent (URL2), which comprise primarily guanidium thiocyanate and phenol, provide a robust means of disrupting cells, protecting nucleic acid and removing proteins, lipids and long-chain polysaccharides. However, the use of shrimp tissue amounts in access of that recommended for any particular nucleic acid extraction method and/or tissue type can result in nucleic acid being either degraded or contaminated with these inhibitors of reverse transcriptase or *Taq* DNA polymerase.

Other PCR inhibitors. Contaminating ethylene diamine tetra-acetic acid (EDTA) and organic compounds such as formalin can inhibit PCR (Dieffenbach and Dveksler 1995). EDTA is commonly present in DNA extraction reagents to chelate Mg^{2+} and thus inhibit RNases and DNases that might degrade nucleic acid. However, as *Taq* DNA polymerase also requires Mg^{2+} for activity, caution is required in extraction methods using ethanol to precipitate RNA/DNA, as without washing the pellet with 70% ethanol, traces of EDTA can remain in residual ethanol. Ethanol itself, however, is well tolerated by PCR at concentrations <10% (Loffert *et al.* 1997).

Formalin is a common component of Davidson's and other tissue fixatives used for shrimp histology and, at low concentrations, is also used in postlarvae (PL) stress tests (Latendresse *et al*. 2002, Samocha *et al*. 1998). As formalin effectively modifies and cross-links protein and nucleic acid, and if stored inappropriately can also generate a strong acid capable of hydrolyzing nucleic acid (Srinivasan *et al*. 2002), histology fixatives should be avoided and stress-tested PL should be washed in fresh seawater prior to nucleic acid extraction.

As potent uncharacterized inhibitors of PCR occur in shrimp eyestalks (URL3), this tissue should be avoided except for larval and postlarval samples, where the eyestalk comprises too little of the total tissue for the inhibitors to be troublesome.

Nucleic acid integrity. Nucleic acid in shrimp tissues can rapidly degrade if samples are collected and preserved using inappropriate methods, resulting in PCR test failure. Repeated freezing and thawing will also cause degradation, as will exposure to strong acidic and basic chemicals (Markham and Smith 1952). To avoid PCR test failure due to chemically-induced nucleic acid hydrolysis, washing of forceps and scissors used for tissue dissection is recommended if bleach is used for instrument sterilization between sampling of individual shrimp. As RNA is generally more susceptible to enzymatic and chemical degradation than DNA, extra care is needed in tissue collection and preservation to avoid failure of PCR tests designed to detect viruses with RNA genomes.

In addition to the precautions needed to obtain high quality nucleic acid, RNA/DNA integrity and the validity of PCR test data is best defended through the co-amplification of an endogenous gene sequence (Sachadyn and Kur 1998). Whilst spiking nucleic acid with an artificial template provides an excellent means of validating the performance of the PCR *per se*, PCR amplification of an endogenous gene RNA/DNA is essential to confirm nucleic acid quality.

Thermal cycler temperature stabilities and ramping rates can also influence PCR efficiency. Temperature overshooting during the DNA denaturation step can be particularly problematic as the half-life of *Taq* DNA polymerase reduces rapidly at temperatures above 94°C (ie. 6 h at 94°C, 2 h at 95°C or 30 min at 96°C) (Lawyer *et al*. 1993). The ideal primer annealing temperature (Tm) also needs to be determined to optimize PCR efficiency and minimize amplification of non-specific products. Moreover, regular calibration of thermal cycling equipment is recommended to ensure performance specifications are met. PCR and equipment performance should also be confirmed by regularly assessing the detection sensitivity of a standardized serially-diluted template positive control.

PCR primer affects. PCR performance can be compromised severely by PCR primer design factors as well as quality, concentration and storage method. Design rules to obtain effective PCR primers are discussed in detail in numerous books (Dieffenbach and Dveksler 1995) and research articles and numerous software are available for their design to meet varied test requirements. Moreover, formal reporting requires the use of either certified commercial PCR kits or methods endorsed by the OIE. Thus, for shrimp pathogen diagnosis, primer design issues are usually not a concern. PCR primers are short oligonucleotides synthesized chemically by the 3' to 5' addition of DNA amidites (Kornberg 1988). Due to inefficiencies at each extension step, a small portion of the oligonucleotides will not be extended, and thus purification by polyacrylamide gel electrophoresis (PAGE) or high performance liquid chromatography (HPLC) is recommended to remove incomplete primers. The presence of incomplete primers would overestimate the concentration of the desired full-length PCR primer, and their lower Tm would provide opportunities for the amplification of non-specific products. Fortunately, most commercial companies now supply PCR primers purified by HPLC or PAGE after elution from Oligo Purification Cartridge (OPC) columns. Precluding primer sequence confirmation by mass spectrometry, sequence analysis of a PCR product can be used to confirm the sequences of both the PCR primers and expected DNA target. PCR primers are best stored at -20°C or lower temperatures as high concentration stocks from which working stocks can be prepared as required. Moreover, tris(hydroxymethyl)aminomethane (Tris) or HEPES buffers containing low (0.1 mM) amounts of EDTA can also be useful in avoiding primer degrading through the action of enzymes or hydrolysis due to fluctuations in pH.

Buffer, dNTP, and enzyme affects. Commercially available PCR buffer and deoxyribonucleotide (dNTP) concentrates as well as *Taq* DNA polymerase, which usually contains preservative agents, can be stored frozen at -20°C and retain good stability for at least one year. However, working dilutions of dNTPs should be stored in small aliquots to minimized thaw/freeze cycles and, as good practice to maintain stability and avoid contamination of reagents, buffer and working stocks of primers should be apportioned and stored similarly in multiple lots.

All of the potential pitfalls in PCR methodology outlined above should be addressed by establishing standard operating procedures (SOPs). These SOPs need to ensure that nucleic acid extraction methods are uniform and produce nucleic acid of adequate quality and quantity and that nucleic acid concentrations are determined so that input volumes can be adjusted to deliver a standardized amount of template into each PCR. Moreover, the SOPs need to ensure that any potential deficiencies in the PCR associated with primers, buffer, dNTPs, enzyme or the thermal cycler are accommodated through the use an

appropriate internal control, a standardized and serially-diluted positive control as well as either nucleic acid extracted from a non-infected shrimp or a water (no template) control. These controls allow performance of the nucleic acid extraction and PCR procedures to be validated and afford information on the possibility of the test giving false-positive or false-negative results.

Limitations of PCR in diagnosing shrimp viruses

Statistical aspects of sampling and target tissue selection affect the accuracy of PCR in diagnosing pathogens in cohorts of shrimp. For example, the fact that nucleic acid concentrations can be increased beyond threshold levels that inhibit PCR defines the tests sensitivity limits. Based on the estimated size of the shrimp genome (~10^9 base pairs, which is equivalent to a mass ~10^9 x 660 g = ~6.6 x 10^{11} g) (Bachmann and Rheinsmith 1973) and thus a molecular weight of shrimp chromosomal DNA of ~6.6 x 10^{11} g/6.023 x 10^{23} = ~1.1 x 10^{-12} g, the maximum number of genome copies that can be placed in a PCR, assuming that inhibition will likely occur above 1 µg (Dieffenbach and Dveksler 1995), is in the order of ~10^{-6} g which equates to ~10^6 chromosomal DNA copies or ~10^6 cells. Therefore, as PCR can theoretically detect a single RNA/DNA molecule, it should be capable of detecting a single viral DNA copy amongst chromosomal DNA from about 1 million cells. However, this assumes that every PCR test is sufficiently sensitive to detect a single DNA molecule and that it can accommodate 1 µg DNA without being quenched. Under more realistic PCR conditions, the detection limit would more likely be in the vicinity of 10-fold less, approaching 1 viral DNA copy amongst DNA from 100,000 cells, which is termed the molecular restriction of PCR in detecting a shrimp virus.

Another limitation of PCR in detecting shrimp pathogens arises when large population sizes need to be assessed, such as when wild broodstock, batches of postlarvae and pond grow-out stocks are tested to estimate pathogen infection prevalence and loads. The example below describes how PCR data for white spot syndrome virus (WSSV) obtained in shrimp hatcheries needs to be interpreted appropriately, rather than in absolute terms not supportable by the testing system. If a pool of 15 PL10 from a hatchery tank test positive for WSSV by PCR, this can only be interpreted as one or more of the PL being infected with WSSV at a load sufficient to be detected by the PCR, not that all shrimp are infected. Correspondingly, a PCR-negative result can only be interpreted as none of the 15 PL10 in the pooled sample being infected with WSSV at a level sufficient to be detected by the PCR. Likewise when broodstock are determined to be PCR-positive for WSSV, as found in case studies (Lo *et al*. 1997), such data is not an absolute predictor that all progeny will become infected. Infection severity and hatchery egg-washing processes will affect the likelihood of WSSV being transmitted vertically to progeny. However, even with only a small portion of infected PL seeded into a pond, they remains a risk of crop losses due to disease and mortalities caused by WSSV.

For this reason, it is important to have a good understanding not only of the PCR test performance and detection sensitivity limit, but also sample sizes needed with large populations to generate data within required prevalence estimates and statistical confidence

limits. Within a population >100000 in size, sampling of at least 150 individuals is required to detect a pathogen with 95% confidence at a 2% infection prevalence threshold (Alday de Graindorge 2002). If only 60 individuals are tested, this confidence interval can only be met for a 5% infection prevalence, and this drops to a 20% or higher infection prevalence when 10 or fewer individuals are tested. The number 150 in a large population size is thus termed the statistical restriction of PCR detection of a shrimp virus.

WSSV loads detected in an infected shrimp by real-time PCR can often be as low as 100 to 1000 DNA copies per 1 million cells. As the detection limit of PCR is about 10 copies per 1 million cells, shrimp with such low-level infections are readily detected. However, samples from several shrimp and larger numbers of PL are commonly pooled to detect viruses at low infection prevalence. For example, for a pool of 100 PL, if one individual possessed a low-level WSSV infection (eg. 500 DNA copies per 1 million cells), due to its dilution with 99 uninfected individuals, viral DNA levels (ie. 5 DNA copies per 1 million cells) would drop below the PCR detection threshold. To avoid over diluting viral DNA and overloading DNA extraction reagent capabilities, with shrimp PL up to ~PL10 or with small pieces of shrimp gill tissue, for example, it is generally accepted that 30 individuals is the maximum that should be pooled for PCR testing. The number 30 is thus termed the restriction of the dilution factor for PCR detection of shrimp viruses.

To accommodate the requirement of testing 150 individuals in a population size >100000 to detect a virus at a ≤2% infection prevalence with 95% confidence, 5 PCR tests can be undertaken using DNA each extracted from tissues pooled from 30 individuals. This, however, increases the cost of virus detection 5-fold and becomes more prohibitive when tested is required for multiple viruses. For example, certification of the SPF status of *P. vannamei* breeding populations requires them to be screened for viruses including WSSV, Taura syndrome virus (TSV), IHHNV, infectious myonecrosis virus (IMNV), and YHV. Although essential for such purposes, the additional cost of testing 150 shrimp per cohort in most hatchery and farm situations will be prohibitive. Moreover, hatchery and farm managers can find it difficult to understand why individuals within a tank or pond cohort should be different or to interpret why multiple samplings of the same cohort could generate conflicting PCR data, even though this accurately reflects the population infection prevalence rather than simply being due to test technical inaccuracies. Furthermore, rigorous testing for viruses down to a low (2%) infection prevalence threshold will be unwarranted in most commercial settings where operational risks are managed on many fronts at many levels. Realistically, PCR test data can only provide a basis for supporting health management strategies to mitigate rather than eliminate all risks of viral disease outbreaks occurring in shrimp farms.

Sampling dilemmas that need to be considered to interpret diagnostic PCR data appropriately are applicable to any diagnostic method. Information on why they can occur has been provided above to help reduce mistrust in PCR-based diagnostic tests that can arise, for example, when different laboratories obtain different results with samples from the same PL batches or when different pools of samples from the same cohort are scored differently in tests performed at the same laboratory. For data consistency and its appropriate interpretation across different laboratories possibly using different PCR test methodologies, there are clear benefits of regular proficiency testing using defined, standardized and coded test samples. However, in real word situations with samples

submitted for virus diagnosis, greater variability in data quality might be expected simply as a result of differences in sample collection and preservation methods as well as in the viral infection status of the stocks from which samples were collected. Whatever the cause, PCR data which are either inconsistent or difficult to reconcile have the potential to generate conflict or mistrust between laboratory and shrimp farm and hatchery managers. The establishment of SOPs in PCR testing laboratories that minimize opportunities for technical inconsistencies, as well as an improved understanding of PCR and why diagnostic anomalies can arise, will help alleviate this mistrust.

PCR can be used to detect shrimp viruses for various purposes. These include the screening of broodstock either before their use in hatcheries or their inclusion in SPF breeding populations, the monitoring of hatchery and farm stocks when there is no evidence of ill health as an early indicator of disease occurring and for rapidly indentifying the cause of a disease outbreak, all require different sampling strategies. In each circumstance, the statistical accuracy of PCR test data must be considered in light of the number of times an individual is screened or the number of individuals within the test cohort that are screened. In large populations (>100000 individuals), samples pooled from 10 to 30 shrimp will generally be adequate to detect viruses at an infection prevalence in the order of 20% and 10%, respectively, with 95% confidence, and this sample size is often recommended in commercial PCR kit protocols. Moreover, when examining for early evidence of viral disease in pond stocks, for example, preferential sampling of shrimp displaying early gross signs of disease, rather than sampling shrimp at random, is likely to increases the chances of accurate PCR diagnosis (Longyant *et al*. 2005).

Due to the various sampling compromises that complicate how PCR data must be interpreted, it is not surprising that building trust and instilling confidence in its diagnostic accuracy has occurred slowly amongst shrimp hatchery and farm managers. However, with a better understanding of sampling and PCR limitations, management actions based on PCR data should facilitate improvements in shrimp culture systems and profitability. By analogy, shrimp culture can be likened to driving a motor vehicle and the wearing of a seat belt. Road accidents are an inevitable fact of life, but wearing a seat belt can substantially reduce the risk of severe injury or death. Similarly, the application of a simple PCR test and appropriate action based on the data can markedly reduce the risk of shrimp crop losses due to viral disease. Every farmer, therefore, has to make a decision to 'buckle up or not', so to speak. Once that decision is made, the farmer then needs to ensure that the seat belt is fastened in the correct way to maximize protection in the event of an accident.

Most shrimp viruses cause systemic infections. Infections with Monodon baculovirus (MBV), hepatopancreatic parvovirus (HPV), and obligate intracellular gram-negative necrotizing hepatopancreatitis (NHP), however, are restricted to digestive tract tissues (Lu *et al*. 2006). To detect these pathogens by PCR requires testing of feces or tissues derived from the hepatopancreas or digestive tract. For systemic pathogens such as WSSV, however, viral infection can be detected in most tissues although levels can vary amongst different tissues. Analyses have identified that gills and pleopods of WSSV-infected shrimp contain more viral DNA than other tissues (Durand and Lightner 2002). For WSSV and other viruses in which tissue infection loads can vary substantially, it is thus important to sample tissue types most appropriate for sensitive PCR detection when screening broodstock and juvenile pond stocks. This concern applies equally for screening of live

feed such as oysters, which are not a host of shrimp viruses but, which as filter feeders, can sometimes be PCR-positive presumably due to ingestion of viruses shed into water or present in other small crustaceans. Thus, samples including oyster digestive system are most likely to result in PCR-positives. However, as PCR only detects nucleic acid, it cannot distinguish between live or dead virus. A PCR-positive result, therefore, does not necessarily imply that feeding the oyster to shrimp will pose a risk of infection.

In PCR screening of hatchery progeny spawned from *P. monodon* broodstock infected with WSSV undertaken by diagnostic service providers in India, data have been accumulated to show that, in order of virus detection sensitivity, postlarvae > egg > nauplii > protozoea > mysis life stages. The reason why PCR detection of WSSV in mysis is problematic, even amongst progeny derived from heavily infected broodstock, remains unknown. However, it has been speculated that this might be due to some cellular structures of this larvae stage being insufficient in mass for WSSV to replicate and accumulate or that the numbers of WSSV-infected cells in nauplii do not expand whilst massive increases in cell numbers have occurred during nauplii growth to mysis. The latter scenario would result in a relative reduction in the amount of WSSV DNA present amongst cellular DNA to levels below that required for reliable detection by PCR. By the time shrimp reach PL stages, cell diversity might better accommodate WSSV replication and thus elevated viral DNA levels would become more readily detectable by PCR. Although this hypothesis has yet to be supported by scientific evidence, available data suggest that care is needed to avoid larval stages that might give misleading results.

The sampling issues discussed in this section can profoundly affect the diagnostic outcome of PCR and highlight common shrimp culture practices that can make interpretation of PCR data confusing and disputable. Through explanation of some of the issues, farm and hatchery managers will hopefully have broadened perspectives as to how PCR can be used most effectively for shrimp pathogen diagnosis as well as more realistic expectations about how it can contribute to managing the risks of production losses due to disease.

Potential problems associated with the use of PCR

Apart from the problems in applying PCR-based pathogen diagnostic methods in shrimp culture systems involving large population sizes and the nature of virus transmission and disease spread, problems inherent to the PCR methodology itself also need to be considered.

Cross-contamination. The PCR method is extremely sensitive due to its ability to specifically amplify >1,000,000 copies of a DNA fragment in a single reaction. Thus, minute cross contamination of virus-free DNA with viral DNA amplified previously by PCR and acquired from the air or contaminated laboratory equipment or reagents, can result in the amplification and detection of a PCR product and the false scoring of a sample as positive. Conventional PCR methods relying on agarose gel electrophoresis to detected amplified DNA are particularly susceptible to cross-contamination problems due to the need to sample an aliquot of the PCR for gel analysis. There is therefore potential for aerosol generation and equipment contamination with amplified DNA during pipetting these reaction aliquots into the gel wells.

False positive results due to PCR cross-contamination can be a major problem for the method. Ideally, a separate room should be used for post-amplification analysis of the reaction by electrophoresis (Dieffenbach and Dveksler 1995). In the event of persistent cross-contamination problems, firstly, pipettes should be cleaned by disassembly, soaking in bleach for 30 minutes followed by extensive washing with clean water, air-drying for one day before reassembly. Secondly, all inexpensive chemicals and disposables such as distilled water, ethanol, tips, and reaction tubes should be discarded and replaced. Thirdly, nucleic acid extraction and PCR reagents should be pragmatically tested in trial PCRs with no added template to confirm that they are free of contaminants. Potential sources of contaminants can be further reduced by the use of ultraviolet (UV) lamps in the post-amplification sampling and electrophoresis room to degrade DNA amplified by PCR. The use of UV lamps, however, requires robust safety measures including staff training to avoid UV-induced eye and skin damage and other potential hazards such as the generation of ozone. Repeated use of UV can also damage certain plastics including those normally used to manufacture gel electrophoresis tanks and tube racks, which should be stored away from UV irradiation and cleaned with detergent rather than organic chemicals such as ethanol.

As no post-amplification procedures requiring a tube opening step are needed and as PCR reagent master mixes employing, for example, No Amperase® UNG (Applied Biosystems Inc.) are available to avoid DNA re-amplification, contamination problems in real-time PCR are reduced significantly compared to conventional PCR methods. However, the cost of real-time PCR reagents and instrumentation is prohibitive, professional maintenance and calibration of instrumentation is often necessary, and data interpretation requires well qualified personnel. For these reasons, the widespread adoption of real-time PCR within the shrimp culture industry has been limited to date and remains mostly available only to large-scale integrated farms and academic and government laboratories.

To monitor for cross-contamination, a no template control (NTC) must always be included in each sample batch tested by PCR. However, use of this control only identifies whether a PCR reagent has been contaminated. In cases when nothing is amplified from the NTC but unexpectedly high numbers of PCR-positives are obtained, it cannot be discounted that cross-contamination might have arisen during nucleic acid extraction or cDNA synthesis. For this reason, it is also useful to incorporate negative control tissue from an uninfected shrimp, or other appropriate sources, in the nucleic acid extraction and cDNA synthesis steps.

Inability to distinguish between viable virus and non-viable viral DNA. PCR amplifies DNA and as such, as long as the DNA is intact, it cannot distinguish between whether the template DNA originated from a live or dead organism or was circulating freely in the environment. For example, a WSSV-positive PCR result from a sample of processed feed might indicate that WSSV-infected tissue was used in its production and that residual WSSV DNA remained present. However, due to the high temperatures used in feed processing, no viable WSSV would remain and the feed would present no risk of infection.

Non-crustaceans used as live feed during broodstock maturation can include, amongst others, polychaetes, squid, and oysters. Taking oysters as an example, both WSSV and TSV have been detected by PCR. However, there are essentially no traceable cases of WSSV infection being transmitted via oysters, whilst transmission of TSV appears to be very

common. By their nature, these two viruses have different tolerances to the oyster digestive system, with TSV being more resistant than WSSV, but PCR data can be confusing as WSSV DNA can remain in the absence of viable virus. The same dilemma exists in PCR analyses of bird droppings where viable and non-viable virus is not distinguishable, and transmission bioassays are required to confirm infectivity (Garza *et al*.1997).

False negatives caused by viral genome variation. Genome mutation rates in RNA viruses in particular, but some DNA viruses as well, are often far higher than in higher order organisms due to viral RNA polymerases lacking proof-reading activity. Any nucleotide mutations, deletions or insertions occurring at PCR primer annealing locations could result in PCR failure or in unexpected non-specific products being amplified. With real-time PCR, mutations existing in TaqMan probe binding sites especially can completely abrogate PCR amplification. As an example of a rapidly mutating virus, a commercial PCR test to detect human immunodeficiency virus (HIV) approved by the US-FDA was found to be unable to detect a newly-discovered strain of HIV due to primer-sequence mismatches (URL4). In the use of PCR, designers of both in-house and commercial kits need to be aware of research examining viral strains and variants so that test primers and conditions can be adjusted appropriately on a regular basis to avoid false negatives arising through virus genome sequence variation.

False positives caused by similar sequences. If virus and host DNA sequences, or sequences between virus types are sufficiently similar, PCR can generate false positive results. The non-infectious IHHNV genome sequence integrated within the genome of some *P. monodon* (Tang and Lightner 2006) and genotypic variants in the YHV complex (Wijegoonawardane *et al*. 2008a; 2008b) are two good examples where cross reactions can occur. However, by the careful design of PCR primers to regions possessing sequence variation, PCR tests have been designed that provide specificity to the virus types of concern and avoid potential false-positive results (Cowley *et al*. 2004).

The amplification of non-specific DNA sequences by PCR can sometimes occur when little or no specific DNA is available, as perfect matching of the PCR primer and a template sequence is not essential for PCR. In general, DNA target mismatching at primer 3' end sequences is most detrimental to PCR amplification efficiency. However, limited mismatches near to the 3' end or elsewhere in the primer sequence are quite well tolerated as these only reduce the primer Tm and thus the stability of primer-template annealing at a set temperature (Dieffenbach and Dveksler 1995). However, in cases where primers have attached inappropriately and amplification of a non-specific product has occurred, this DNA incorporating the PCR primer will provide a perfect match for its subsequent specific amplification by the same primer.

In addition to good PCR primer design, the application of nested PCR can enhance detection sensitivity and specificity by using a second primer pair targeted to sequences internal to primary PCR product. In a similar manner, use of real-time PCR methods employing a TaqMan probe, molecular beacon, or light cycler probe can similarly improve specificity as well as provide a means of quantifying infection loads and reducing the chances of technical errors. These factors in addition to its inherent speed, computer generated data format, avoidance of cross-contamination and the need for gel-based PCR product analysis, and its amenability to high through-put application are compelling reasons why most diagnostic PCR systems for human diseases approved by the US-

FDA are now based on real-time PCR formats. Regrettably, uptake of real-time PCR for application in the shrimp farming industry lags well behind human medicine as well as other aquaculture industries.

Proficiency auditing of PCR laboratories

Continued use of PCR for pathogen detection will remain important to minimizing the risks of disease and production losses in shrimp aquaculture. Because of these risks, many larger shrimp hatcheries and farms have well-established PCR testing laboratories. However, the managers entrusted with making decisions to maximize production outcomes often lack a technical understanding of why PCR can sometimes generate equivocal data through inadequacies in sample collection methods or test procedures. Because of this, whenever any doubt about PCR data arise, laboratory deficiencies are often the first to be blamed irrespective of whether the problem occurred due to inadequacies in sampling rather than the performance of the PCR. Such situations can also be compounded by the often competitive and secretive behaviors of shrimp hatcheries and farms and an unwillingness to invest substantially in staff training and automated nucleic acid extraction and real-time PCR instrumentation to avoid human error and contamination problems. As a result, improvements in testing proficiency have mainly been incremental and as suspicions and queries occur frequently, defending test data can become habitual for PCR testing laboratories. This defensiveness can lead to closed minded attitudes that hinder robust discussions around ways to improve operational management and quality assurance systems within such laboratories important to establishing trust in the data generated.

Measures that can be implemented to assure PCR data quality and reduce controversy, although difficult, are worthy of investment to maximize the potential benefits that such testing can provide. Discussion below relates to Section IV and focuses on some of the problems most frequently encountered in PCR testing laboratories. Auditing of PCR testing laboratories for proficiency falls broadly under management and technical requirements.

Management requirements. Good management of a PCR testing laboratory primarily requires professionally qualified personnel to plan and manage staff and laboratory systems and ensure appropriate reagent storage, equipment calibration, sample labelling, reporting procedures, data filing, data interpretation, and staff training. The ISO/IEC17025 standard provides many valuable concepts for service laboratory management (URL5). It might not be practical to demand that PCR testing laboratories servicing the shrimp culture industry comply absolutely with all procedures recommended in this standard. However, the concepts it conveys can serve as instructional material for establishing laboratory operation procedures needed to consistently generate high quality data.

Technical requirements. To generate reliable and consistent high quality data, a PCR testing laboratory requires highly competent staff, robust methodologies and regularly calibrated equipment to ensure its operation within defined parameters. Staff training endeavors to negate human error in sample labeling, DNA extraction, PCR and electrophoresis that can be major sources of data inconsistency. To address this, the ISO/IEC17025 standard provides guidelines for personnel training in laboratory safety,

emergency procedures and equipment operation as well as more specifically in technical areas associated with sampling principles, PCR principles and basic biostatistics. With regard to methodologies, it is imperative that SOPs be established and validated and revised as necessary to ensure consistency in PCR data generation. Ideally, suppliers of the key reagents and enzymes needed for PCR should also possess ISO9001, or better still, ISO13485 accreditation. The use of commercial PCR kits endorsed by the OIE is another means of ensuring data consistency. If such commercial kits are bypassed for in-house PCR tests, their sensitivity, specificity and diagnostic accuracy should be verified in direct comparisons with OIE-endorsed tests. It is also mandatory that equipment undergo regular maintenance and calibration to ensure that test performance is not compromised.

Correct and reliable PCR data should be expected from laboratories in which a total quality system has been implemented based on high levels of personnel training and robust operation procedures, management models, and customer service initiatives as recommended in the ISO/IEC17025 standard and certified by a verifying organization. However, this ideal situation can only, as yet, be regarded as a future goal of most PCR testing laboratories servicing the shrimp culture industry, and thus more practical and easily accommodated interim measures are needed to improve data consistency. Amongst others, inter-laboratory calibration and proficiency projects have been undertaken and provided positive outcomes. Whilst the ISO/IEC17025 standard certifies that data will meet certain minimum quality standards, inter-laboratory calibration can identify laboratories that are deficient and recommend procedures that can be implemented so that quality standards can be lifted above the minimum required.

For inter-laboratory PCR proficiency testing, a reference laboratory will distribute coded standardized samples to participating laboratories for analysis and scrutinize data returned. The results of this process can be used to inform laboratories of their performance in correctly diagnosing samples so that improvement measures can be implemented if necessary. At present, Dr. Donald Lightner's team at the University of Arizona, Tucson, USA, is coordinating proficiency testing of PCR laboratories servicing the shrimp culture industry in Mexico (Pantoja and Lighter, 2006), and Dr. Peter Walker's team at CSIRO Livestock Industries, Geelong, Australia is coordinating similar testing of PCR testing laboratories servicing the shrimp culture industries in India and Vietnam (URL6). These inter-laboratory proficiency tests should highlight current levels of PCR laboratory proficiency in these countries and be instructive on their broader use to improve data consistence between laboratories.

Isothermal amplification alternatives to PCR

Commercial PCR tests for shrimp pathogens have been marketed and used successfully for over 10 years. However, other RNA/DNA detection systems based on isothermal amplification methods such as loop-mediated isothermal amplification (LAMP) (Notomi *et al*. 2000) nucleic acid sequence based amplification (NASBA) (Compton 1991) and rolling circle amplification (RCA or RAM,) (Fire and Xu 1995) have more recently been established and demonstrated to be useful in diagnosing pathogens including shrimp viruses. These methods provide some advantages over PCR by only requiring a constant

temperature system, such as a heated water bath, rather than more expensive thermal cycling equipment. As the isothermal amplification methods can detect nucleic acid at sensitivities approaching that of PCR-based methods, they offer possibilities for cost effective onsite use for pathogen screening at shrimp hatcheries and farms.

Will isothermal amplification methodologies such as LAMP become widely adopted or supplant PCR as the method of choice for shrimp virus diagnosis? The answer to this question requires examination of the benifets and disadvantages of each platform and how they might best be applied. Conventional PCR requires commonly available reagents, two short oligonucleotide primers, a thermally-stable enzyme, thermal cycling equipment and an agarose gel electrophoresis system. The requirements for LAMP are similar except that thermal cycling equipment is not needed and that it employs 4 to 6 longer primers targeted to 6 to 8 regions in the DNA to be amplified. The LAMP method is, therefore, more susceptible to sequence mismatches between the target DNA and primer generating a false negative result, and far more complicated than PCR to apply for the multiplexed detection of different viruses or viral strains/genotypes. Similarly to PCR, LAMP detection of RNA requires its conversion to cDNA using reverse transcriptase. In contrast, NASBA provides an extremely efficient method for directly amplifying RNA in reactions incorporating the 3 enzymes, reverse transcriptase, RNaseH and T7 RNA polymerase. However, whilst NASBA reactions also use commonly available reagents and only require brief heating at 100°C and amplification at 41°C, detection of amplified RNA requires the use of Northern blotting RNA hybridization, which is complex and time consuming, and only provides a rough estimate of infection loads. RAM can use various enzymes such as ϕ29 or *Bst* DNA polymerase in combination with *E. coli* DNA ligase and relies on a process whereby a short (>100 nt) ssDNA circle is generated and can be replicated repeatedly following its hybridization to a specific DNA primer. In diagnostic applications, this ssDNA circle will be derived from the pathogen being detected. Although as equally sensitive as PCR, the RAM method generates DNA products that vary in size, are not readily quantified and that could be due to the amplification of non-target DNA similar in sequence to the pathogen being detected (Kuhn and Khmelnitsky 2005). Whilst the three isothermal amplification methods described above can afford high detection sensitivities similar to PCR in the order of 1-10 DNA copies/reaction, in terms of complexity and data interpretation when used for pathogen diagnosis, PCR is far simpler, more convenient and more easily interpreted. Moreover, unlike the isothermal amplification methods, PCR provides for excellent quality assurance through the use of controls to ensure test specificity and to avoid and/or identify false negative or false positive results.

As with PCR, isothermal amplification methods can be adapted to automated real-time fluorescence detection systems. Whilst real-time PCR can accurately resolve small differences (<2-fold) in template number (Pfaffl 2001) over a 6 order linear range (Bustin 2000), isothermal amplification can only resolve larger (>10-fold) differences in template number over a 4 order linear range. Moreover, the enzyme and/or primer systems utilized by isothermal amplification methods are more complex than PCR and offer limited capabilities for amplifying and discriminating several targets within a single reaction. They are also less amenable to differentiating closely-related pathogen variants, as unlike PCR, they provide no ready means of amplicon sequence analysis. The characteristics of isothermal and PCR amplification methods are summarized in Table 3.

Table 3. Characteristics of isothermal and PCR amplification methods highlighting the relative advantages of each test

	PCR	*Isothermal amplification*
Test design complexity	low	high
Specificity/sensitivity	good	good
DNA/RNA detection	yes	yes
Quantification	yes	no
Multiplex application	yes	no
Strain typing ability	good	moderate

The primary advantage of the isothermal amplification methods over PCR is that they only require a method of maintaining a constant temperature rather than expensive thermal cycling equipment. However, this advantage is negated by their requirement for longer and more complex primers, which for tests for multiple pathogens could prove to be as expensive as purchasing a thermal cycler. Moreover, the enzymes and reaction components used in isothermal amplification methods can be more complicated and less stable than those used in PCR. For example, NASBA requires the use of ribonucleotides (rNTPs) which are more expensive and less stable than dNTPs used for PCR and *E. coli* DNA ligase activity requires NADPH as co-factor, thus adding to the complexity of the reaction buffer (Wilkinson *et al*. 2001). Furthermore, quality control (QC) and quality assurance (QA) measures for commercial isothermal amplification kits would be far more difficult to incorporate than they are for PCR.

The susceptibility of PCR to contamination problems and its inability to distinguish viable from non-viable pathogens are shared by the isothermal amplification methods. Whilst real-time PCR can assist in avoiding contamination problems, equipment and reagent costs can be restrictive. In contrast, isothermal amplification systems are less prone to contamination problems and fluorescence detection equipment is currently ~10-fold less expensive than required for real-time PCR. As longer primers are used, false positives might occur less frequently and minor sequence variations amongst viral strains less likely to manifest as false negatives. There are, therefore, grounds for these tests becoming more widely adopted for shrimp pathogen detection. Irrespective of these technical advantages of isothermal amplification methods, PCR currently is the virus diagnostic tool of choice, and is likely to remain so due to its reaction stability, specificity and sensitivity, its high throughput capabilities and the ready availability of commercial detection kits. Indeed it is unlikely that any substantial advantages would be derived from laboratories with well established PCR testing capabilities switching to the use of isothermal amplification methods. However, in circumstances where access to skilled laboratory technicians and the cost of PCR equipment and kits, are prohibitive, the adoption of commercially available isothermal amplification kits could provide a viable alternative tool for managing the health of cultured shrimp. With the recent availability of commercial real-time PCR kits for shrimp pathogen diagnosis, DNA amplification-based diagnostic platforms can be selected as viewed to be most appropriate for application to the many and varied shrimp culture systems being utilized in developed and developing countries around the world.

Conclusions

PCR is a very powerful tool for providing accurate and sensitive diagnostic information to manage shrimp health. However, as outlined in this chapter, inappropriate management actions based on misinterpreted PCR data needs to be avoided through a better understanding of its capabilities and limitations for shrimp culture applications. Armed with such information, farm and hatchery managers will be more aware of how PCR can be implemented best to reduce the risks of production losses due to disease. It needs to be remembered that PCR is simply a robust and sensitive means of detecting RNA/DNA of viruses and other pathogens with molecular and statistical limitations, rather than a panacea for solving all disease problems afflicting the shrimp culture industry. Ongoing research is needed to continually update knowledge of viral strain variants derived either through mutation or recombination that might result in PCR generating false-negative or false-positive resuts. Moreover, those engaged in providing PCR testing services would benefit from regularly updating their knowledge of advances in PCR technology and from implementing standardized operating systems for quality assurance. As specialized equipment reduces in cost, automated nucleic acid extraction and real-time PCR systems that minimize the potential for cross-contamination and human error, as well as increase test throughput, are likely to become more widely adopted. With such innovations in these technologies and the availability of reasonably priced equipment, wise investments in these high-throughput systems could tremendously increase the impact of PCR in circumventing disease problems in cultured shrimp.

Currently the onus is on PCR laboratories to avoid all possible sources of test inaccuracies or failures and provide appropriate and statistically supported interpretations of what the data means to managers of shrimp hatcheries and farms. Such laboratories often work under extreme time pressures to generate results, but often also lack resources and a respect for the difficulties they face in providing accurate information upon which health management decisions can be made. Hopefully the shrimp culture industry will acknowledge the value of PCR testing laboratories and commercial services in the near future so that their contributions can be acted upon without compromise or conflicts to ultimately improve its profitability and long term viability.

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