

13. Nucleic acid based methods in food borne pathogens

Ahamed Basha, K and Madhusudana Rao, B

ICAR-Visakhapatnam research centre of Central Institute of Fisheries Technology(CIFT),
Pandurangapuram, Visakahapatnam-530003

Introduction

Aquaculture is one of the fastest growing food-producing sectors playing an immense role in providing food security and livelihood. Although continuous efforts are being employed across worldwide to prevent the infectious diseases of fish and shellfish, still new outbreaks continue to be recorded creating major constraint to the aquaculture industry. Foodborne diseases have turned out to be a major public health problem worldwide due to significantly rise in incidence of foodborne diseases over the last 20 years. Pathogens causing foodborne diseases are often referred as foodborne pathogens and they include bacteria, viruses, fungi and parasites. The common foodborne pathogens responsible for the majority of foodborne disease outbreaks are *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Staphylococcus aureus*, *Salmonella enterica*, *Bacillus cereus*, *Vibrio* spp., *Campylobacter jejuni*, *Clostridium perfringens*, and Shiga toxin-producing *Escherichia coli* (STEC). In general, foodborne diseases are caused by the consumption of food or water contaminated pathogens or their toxins. Therefore, to ensure safe food and to reduce the incidence of foodborne pathogens, analyzing the food samples free from these pathogens is highly essential. Earlier to the introduction of molecular methods, detection of foodborne pathogens relied on culture dependent approach i.e isolation of the causative organism in media followed by examination of phenotypic or serological properties of the pathogen. Most of conventional methods are powerful and trustworthy; however, they are usually laborious, complex and time consuming. In addition to that, prior knowledge about the pathogen in the sample is required and it is well known that not all pathogens are culturable, including viruses and toxins. Typically, conventional methods require 2 to 3 days for primary identification of pathogen and more than a week for its species confirmation. In recent times, various rapid methods with high sensitivity and specificity have been practiced to overcome the limitations of conventional methods for their speedy detection and identification of foodborne pathogens. The advantage of rapid detection methods particularly in food industry, are essential as they are able to detect the presence of pathogens in raw and processed foods immediately. Rapid methods are also sensitive enough to detect pathogens that present in low numbers in the food. Sensitivity related to diagnostic tool is important because a single pathogen present in food has the risk to cause infection. Rapid methods are more time-efficient, labor-saving and able to reduce human errors. Nucleic acid-based methods detect specific DNA or RNA sequences in the target pathogen by hybridizing the target nucleic acid sequence to a synthetic oligonucleotide (probes or primers) which is complementary to the target sequence.

In diagnostics, detection of nucleic acids in a pathogen depends highly on the separation of nucleic acids from the sample, the quality and amount of the target sample. Nucleic acids methods such as simple polymerase chain reaction (PCR), multiplex polymerase chain reaction (mPCR), real-time/quantitative polymerase chain reaction (qPCR), etc, used frequently in detection of foodborne bacterial pathogens are elaborated in this literature.

1. Basic PCR

Presently, PCR has evolved as one of the most important molecular diagnostic methods used frequently for the detection of foodborne pathogens. PCR is a highly sensitive and robust technique, derives its name from one of its key components, a DNA polymerase used to amplify a portion of DNA by *in vitro* enzymatic replication. Due to its speed, limit of detection (LOD), sensitivity and specificity to the target pathogen, PCR is considered to be an alternative method to the culture-based detection techniques. The PCR was first discovered by Kary Mullis during 1983 and invention of this technique earned him the Nobel Prize in Chemistry in 1993. As published by Kary Mullis in 1990, PCR was described as incarnadine, visionary and self-indulgent. The principle involved in PCR is denaturation, where the double-stranded DNA (dsDNA) is separated into single stranded DNA (ssDNA), followed by primers annealing to the complementary sites on the ssDNA. The primers are extended by the DNA polymerase by adding one complement nucleotide after another in array to generate copies of the original DNA template. PCR works by amplifying a specific target DNA sequence in a cyclic three steps process. Initially, the target dsDNA is denatured into ssDNA at high temperature. Further, two single-stranded specific synthetic oligonucleotides (primers) forward and reverse primer will anneal to the ssDNA strands. This is followed by the extension process whereby the primers complementary to the single-stranded DNA are extended using deoxyribonucleotides and a thermostable DNA polymerase. The three steps (denaturation, annealing and extension) are repeated multiple times in a thermocycler, where each synthesized DNA strand acts as template for the synthesis of new DNA strands. Each cycle will be generating double the initial target number, when appropriate conditions are used. The PCR amplified products (i.e. amplicons) are visualized by gel electrophoresis. The gel is stained with a fluorescent dye such as ethidium bromide or SYBR Green that binds to the PCR products.

Essentials of PCR

To set up a PCR reaction, several components are required for generating amplicons

a. DNA polymerase

Taq was the foremost thermostable enzyme used for PCR and emerged as one of the most important reagents in commercialized molecular biology. The selection among the enzymes has to be determined by the purpose of the experiment. In 1956, Arthur Kornberg isolated the first DNA polymerizing enzyme, now known as DNA polymerase I and this won him the Nobel Prize in 1959. Presently, broad choice of enzymes are discovered to catalyze the DNA template but variability in their fidelity (error rate per nucleotide), efficiency and ability to synthesize large DNA products needs to be known before selecting the suitable enzyme. Thomas Brock, microbial ecologist reported new species of thermophilic bacterium, *Thermus aquaticus* was isolated from the outflows of Yellowstone National Park and Taq DNA polymerase was isolated from the same organism in 1976. Across the globe, Taq DNA polymerase is often used and acts as workhorse for routine amplification of small DNA segments. To perform a routine PCR, Taq polymerase of 0.5-2.5 units per normal 25-50 μ l is ideal choice of enzyme. Certainly, it has a 5'→3' exonuclease activity and despite variations in 3'→5' exonuclease "proofreading" function, Taq is widely used in PCR labs. Processivity (Extension rate) is speed at which the nucleotides are added to DNA molecule. Manufacturers supply Taq DNA polymerase in storage buffer with 50% glycerol. It is recommended that Taq DNA polymerase available in tube should never reach to room temperature. As the solution is viscous in nature and difficult to pipette precisely, allow the microcentrifuge tube with enzyme to centrifuge at 4°C for 10-15 seconds using positive displacement method (piston is in direct contact with the liquid). Never release the enzyme into water free from buffer to avoid its denaturation. The optimal elongation temperature of Taq DNA polymerase is 75–80 °C, although lower temperature range (68–72°C) commonly used. Taq DNA polymerase is produced in native form *Thermus aquaticus* as well as its recombinant form produced by *Escherichia coli*. For optimal elongation, divalent magnesium ions are necessary. Higher concentrations of Taq DNA polymerase might produce nonspecific products. When blood is haemoglobin, lactoferrin of the blood plasma and Ca²⁺ are reported to inhibit the PCR amplification. In broad prospective, amplification efficiencies of Taq are reported to be 80% at targets shorter than 1kb when template CG content is between 45 to 56% and amplicons efficiency commonly decrease with increasing amplicon size exceeding 1 kb. Covalent linking of the polymerase domain with the *Sso7d* a small, positive charged DNA binding protein from *Sulfolobus solfataricus* enhances the performance of Taq when target amplicons are >1kb. Few newly isolated enzymes (*Vent* and *Pfu*) can perform 3'→5' exonuclease proofreading function unlike Taq, which does not have this editing function. Eventhough its low fidelity rate is of concern (2x10⁻⁴ error/bp per duplication), high thermostability, durability and reliability made Taq DNA polymerase a vital reagent in PCR reactions.

Table1. Characteristics of Common DNA Polymerases for PCR

DNA polymerase		
	<i>Taq</i>	<i>Vent/Tli (Pfu is similar)</i>
Half-life @ 95°C	~ 90 min	~ 420 min
3'→5' Exonuclease	No	Yes
5'→3' Exonuclease	Yes	No
Extension rate (nt/sec)	75	>80
Error rate (errors/bp)	2×10^{-5}	4×10^{-6}
Resulting ends	3' A	>95% blunt
Application	Standard amplification of target sequences	Target amplification for cloning blunt-ended fragments requiring DNA sequence fidelity

b.Synthetic oligonucleotides or primers

Among many factors, design of oligonucleotides plays a major role in affecting specificity and efficiency of the PCR reaction. For a success or failure of a PCR protocol, selection of the oligonucleotide is often critical for the overall success of a PCR experiment. In this regard to obtain desired products with high yield and to suppress non specific amplification, careful design of primers is highly essential. For a standard PCR reaction, typically 0.1 μ M-0.5 μ M of each primer (forward and reverse) is sufficient enough for minimum amplification of 30 cycles to yield 1kb segment DNA. Typically, each primer contain 17-30 nucleotides in length with approximately same number of four bases as well as balanced G and C distribution (40-60%). Oligonucleotides of 20 bases with a 50% G + C content generally include T_m (T_m = defined as the dissociation temperature of the primer/template duplex) values in the range of 56-62°C. The GC content and T_m within a primer pair must be well matched. For primers shorter than 20 bases, an estimate of T_m can be calculated as $T_m = 4(G + C) + 2(A + T)$. Poorly matched primer pairs can be less efficient and specific because loss of specificity arises with a lower T_m and the primer with the higher T_m has a greater chance of mispriming under these conditions. If a higher temperature is used, the primer of the pair with the lower T_m might not be functioning. This matching between GC content and T_m is vital when selecting a new pair of primers. More the primer length and higher can be the specificity. The primary molar ratio among the primers and the genomic target sequence is at least $10^8:1$, for a genomic DNA, containing 1 μ g of template DNA (3×10^5 copies of autosomal genes). Care has to be taken to avoid primer-dimer formation and secondary structure formation such as hairpin formation, primers must be designed free from selfcomplemntarity and intercomplemntarity sequences not exceeding >3bp in length. The annealing temperature followed in PCR cycle should be approximately 5°C lower than the melting temperature of

primers. The proportion of the primer to template is also important regarding the specificity of PCR. If the ratio primer to template is too high, PCR is more prone to generate nonspecific amplification products, and high chances of primer dimers. In order to avoid formation of hairpin structures and primer-dimers, more than three G or C nucleotides at the 3'-end of the primer should be avoided because of change in free energy (ΔG) of terminal GC bases. However, if the ratio primer to template is too low, efficiency of PCR is greatly compromised. During designing of primers a four di-nucleotide repeats acceptable in an oligo repeat (di-nucleotide occurring many times consecutively) more than four should be avoided as they can misprime eg: ATATATAT. Primers containing 5 or more long runs of a single base should generally be avoided as they can misprime, eg; AGCGGGGGATGGGG has runs of base 'G' of value 5 and 4. Various computer programs are available to optimize the design, selection of a good primer.

c. Deoxynucleoside triphosphates (dNTPs)

Deoxynucleotide triphosphates (dNTPs) are crucial building blocks of DNA and RNA, and their equimolar concentrations in PCR mixes are required to generate new DNA. The four individual deoxynucleotides making up a DNA sequence (i.e. deoxyadenosine triphosphate, dATP; deoxythymidine triphosphate, dTTP; deoxycytosine triphosphate, dCTP; and deoxyguanosine triphosphate, dGTP) are usually added at a concentrations of 200-250 μM . Higher levels of dNTPs ($>4\text{mM}$) are inhibits PCR reaction as they sequester with Mg^{2+} . dNTPs are supplied in stocks must be free from PCR inhibitors such as pyrophosphates and are chemically stable with slightly alkaline stored at -20°C . Vials with dNTP solutions recommended to be centrifuged followed by thawing to minimize the changes in their concentrations.

d. Divalent cations

Generally, thermostable DNA polymerases require the free divalent ions such as magnesium (Mg^{2+}) as a cofactor during the reaction process. The Mg^{2+} ion forms complex with dNTPs, primers and DNA template and plays major role in yield of PCR product by acting as a catalyst. The optimal concentration range of MgCl_2 recommended is 1.5mM-3mM for PCR reaction. In few cases, it is observed that upto 5mM is also reported to decrease non specific amplification. Too few Mg^{2+} ions result in a low yield of PCR product. However, increased concentrations of Mg^{2+} will also decrease the specificity and fidelity of the DNA polymerase. Lower Mg^{2+} concentrations are desirable when fidelity of DNA synthesis is critical. The concentration of MgCl_2 should be used empirically, from 1 mM and escalating to 0.1 mM, until a sufficient yield of PCR product is obtained. If the DNA samples contain EDTA or other chelators, negatively charged PO_4^{-3} ions might sequester Mg^{2+} , therefore MgCl_2 concentration in the reaction mixture should be raised proportionally. Few cations such as Mn^{2+} works less efficiently as compared with Mg^{2+} and cations like calcium quite ineffective.

e. Buffer

It offers a suitable chemical environment for optimum activity and stability of the DNA polymerase. Standard PCR buffer with 50mM KCl works well for amplification of DNA >500bp. The major components of PCR buffer include Tris-HCl, potassium chloride (KCl) and magnesium chloride (MgCl₂).

f. Template

The quality and purity of DNA will have a considerable effect on the likelihood of a successful PCR reaction. The mass of purified nucleic acids in solution is calculated at 50 µg/ml of double stranded DNA or 40 µg/ml for either RNA or single stranded DNA at an OD₂₆₀ =1.0. The common inhibitors in PCR are contaminants passed through DNA extraction and should be carefully avoided. Protein, RNA, organic solvents, and detergents are the common DNA extraction inhibitors of PCR. Ideally, the ratio of OD_{260/280} is between 1.8-2.0 and OD_{260/280} lower than 1.8-2.0 are indicative of protein and/ or solvent contamination will be problematic for PCR. Practically, genomic (both eukaryotic and prokaryotic), plasmid, phage DNA, amplified DNA, cDNA, mRNA in circular and linear are suitable substrates for PCR. In general, the efficiency of PCR is greater for smaller size template DNA (i.e., earlier amplified fragment, plasmid, or phage DNA) than high molecular (undigested eukaryotic genomic) DNA. Typically, 1 µg of genomic DNA (3x 10⁵ copies) of autosomal genes is required for a PCR reaction. For Yeast, bacterial genomic DNA and plasmid DNA 10 nanogram, 1 nanogram, 1 picogram quantities are used per reaction.

g. Distilled Water

Nuclease free distilled water in sterile condition is used to make up the desired volume for the reaction.

h. PCR Enhancers

In order to increase specificity and yield few enhancers mentioned are included in the reaction

DMSO (dimethylsulfoxide) (1%-10%),

Betaine (1-2 M)

Polyethylene glycol (PEG) 6000 (5%-15%)

Glycerol (5%-20%)

Non-ionic detergents:

Formamide (1.25%-10%)

Bovine serum albumin (10-100 µg/mL)

1.1 PCR programming

PCR reaction includes 3 steps, depending on the product size, 30 to 40 cycles are repeated. The three steps (denaturation, annealing and extension) are constantly multiple times, each synthesized DNA strand acts as template for the synthesis of new DNA strands.

Thermocycler: PCR reaction is carried out in an automated cycler, where simultaneous heating and cooling of the tubes with the reaction mixture in a very shorter duration. The most common thermal cyclers used in laboratories today rely on Peltier technology. Peltier devices are solid-state heat pumps that use a type of semiconductor called peltiers. When a low voltage direct current is applied to one side of this device, heat will travel in the direction of the current, thereby making one side of the block hot and the opposite end of the block cold. Thus, heat and cold can be directed from one side of the block to the other by simply changing the direction of the current. Few thermal cyclers contains a *gradient* function, which can sets up a range of temperatures across the block for a given step of the PCR reaction. A specific range of gradient temperatures can be set by the user to run multiple temperature profiles concurrently for optimizing the cycling conditions (e.g., a gradient of diverse annealing temperatures) for a given set of primers. From a single DNA stand, over 1 trillion copies are generated from a 40 cycle reaction.



Thermal cycler

Initiation

The general formula starts with an initial denaturation step at 94°C to 98°C depending on the optimal temperature for DNA polymerase activity and G-C content of the template DNA. This interval may be extended up to 10 min for GC-rich templates. A typical reaction will start with a one minute denaturation at 94°C and any longer than 95°C for 3 minutes may inactivate the DNA polymerase, destroying its enzymatic activity. The initial denaturation results in activation of DNA polymerase and denatures sufficient number of contaminants.

a. Denaturation

The next step is to set the thermal cycler to initiate 25 to 35 rounds of a three-step temperature cycle. Increase in the number of cycles more than 35 will produce greater quantity of PCR products and too many rounds often results in the enrichment of undesirable secondary products.

The crucial step in PCR reaction is denaturation, the double strand melts into single stranded DNA. Incomplete denaturation of DNA results in inefficient utilization of template during the first amplification cycle leading to a poor yield of PCR product. Usually denaturation for 0.5–2 min at 94–95°C is sufficient. Denaturation time may be increased up to 3–4 minutes, if the amplified DNA has a very high GC content. Alternatively additives such as glycerol (up to 10–15 vol.%), DMSO (up to 10%) or formamide (up to 5%) may be used to facilitate DNA denaturation.

b. Annealing

This is one of the most important steps in cycle to correctly regulate the reaction. The temperature for this step can differ significantly based on the primer composition. Usually the optimal annealing temperature is 5°C lower than the melting temperature (T_m) of primer template for 0.5–2 min is usually sufficient. The closer to the T_m of the primer, the more stringent the conditions for the reaction (i.e. there will be less nonspecific product). In order to overcome nonspecific PCR product, annealing temperature should be optimized gradually increasing by every 1–2°C. During annealing step, primers are wiggling around and ionic bonds are constantly formed and broken between the single stranded primer and the single stranded template. As soon as few bases started building up, the ionic bond is so strong between the template and the primer which does not break anymore. Higher annealing temperatures may result in no amplification due to inability of the primers to anneal to the target sequence. Care should be taken during designing the primer to eliminate many of these problems.

c. Elongation/Extension

The length of time for the elongation step is directly proportional to the length of the amplicon. The recommended temperature of extension is 70–75°C as the rate of new strand DNA synthesis by *Taq* DNA polymerase is highest at this temperature. For example, 30 sec is sufficient for shorter amplicons (100 to 500 bp) whereas amplicons of >1 kb will require elongation times of 1 min or more. For a 500 bp of DNA synthesis, 30 sec elongation time works satisfactorily in a standard PCR. The optional extending time is 1 min for the synthesis of PCR fragments up to 2 kb and further increased in 1 min for each 1000 bp may be required. *Taq* polymerase polymerizes at a speed 1-1.5 kilobases per minute at 72-78°C. Primers that are on positions with no exact match don't give an extension of the fragment. The polymerase functions by adding

up complementary dNTP's to the target sequence/template from 5' to 3', reading the template from 3' to 5'. Because both strands are copied during PCR, there is an exponential increase in the number of copies of the gene. The number of PCR cycles depends on the quantity of template DNA in the reaction mix and on the expected yield of the PCR product.

Cycle number

Generally, 30 to 35 cycles of PCR are adequate to amplify enough products for its visualization on a gel. Considerably increasing the number of cycles does not significantly increase the amount of amplicon. In fact, greater cycle numbers can increase the chance for nonspecific amplification.

Final Extension

The final elongation step is often kept for 5 to 7 min to ensure that the PCR products amplified are full length and to fill-in the protruding ends of newly synthesized PCR products. Also, during this step, the terminal transferase activity of *Taq* DNA polymerase adds extra "A" nucleotides to the 3'-ends of PCR products. This step ensures any remaining single stranded DNA is fully extended after last PCR cycle and final holding step keeps the PCR products at hold for 4-15°C for indefinite time keeping the products for short term storage.

Amplification conditions:

The following condition is a generic profile that can be modified based on the specific requirement of the primers, template, and size of the amplicon.

Initial step: 2 min 95 °C (denaturation)

35 cycles: 15 sec 95 °C (denaturation)

30 sec 55 °C (annealing)

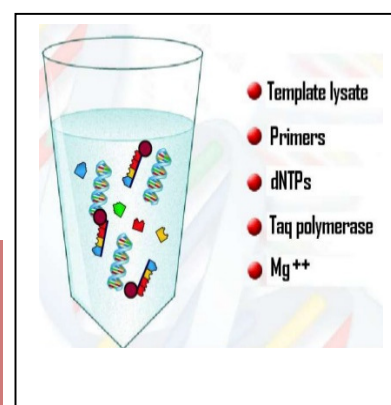
60 sec 72 °C (extension)

Final elongation: 5 min 72 °C (extension)

Final step: indefinite 4 °C (hold)

Table 2. PCR reaction mixture

Components	Final concentration	Per tube volume	Master mix 12 tubes
10× PCR buffer MgCl ₂ -free	1X	5 µl	60 µl
25 mM MgCl ₂	1.5 mM	3 µl	36 µl
10 mM dNTP mix	0.2 mM each dNTP	1 µl	12 µl
10 µM forward primer	1 µM	5 µl	60 µl



10 μ M reverse primer	1 μ M	5 μ l	60 μ l
5 U/ μ l <i>Taq</i> DNA polymerase	0.025 U/ μ l	0.25 μ l	3 μ l
Sterile, nuclease-free H ₂ O		up to 50 μ l (30.75 μ l)	369 μ l
>100 ng/ μ l template	2 ng/ μ l	1 μ l	-

The PCR reaction can be standardized and optimized either by altering conditions such as the denaturing time, annealing time and temperature, Mg²⁺ ion concentration, extension time and temperature, and the individual quantity ingredients in reaction mixture. The final product is visualized under U.V transilluminator to know the status of target gene amplification.

Reaction controls

A positive and negative control in a PCR reaction confirms the validity of the experimental result. The positive control typically contains a template of known sequence and concentration which substantiates the efficiency of the PCR master mix reagents and primers. A negative control includes all the PCR reagents except a template. This control confirms that the reagents used in the experiment are free from template DNA contamination. Typically a negative control is set for every master mix used in a PCR reaction. If amplification is observed in the negative control, the experiment is said to be invalid. The whole PCR experiment needs to be repeated, to determine the source of contamination.

1.2 GOOD PCR PRACTICES

Most common causes of contamination include pipet tips, pipettors, stock solutions and PCR tubes. The most common method of contamination is the improper handling of post-amplification products. The most effective and least costly approach for conducting high-quality PCR-based experiments is achieved by following good laboratory practices.

1. Use a separate set of pipettors with sterile pipett tips and tubes especially for setting up a PCR reactions prior to amplification. Positive-displacement pipettors or aerosol resistant tips will yield best results.
2. The common laboratory reagents such as sterile distilled water, Tris buffer and magnesium salt stocks for setting up PCR must be maintained very close to PCR set up reaction.
3. Constantly wear gloves in the setup area, but never wear them outside of this area, especially where amplicons are released.
4. Wipe down the surfaces of work area, pipettors and outside of tip boxes with a 10% bleach solution to remove residual nucleic acids from surfaces.

5. Separate the area of agarose gel electrophoresis for analysis of PCR products and PCR set up.
6. PCR cabinet or workstation acquisition can be very useful.

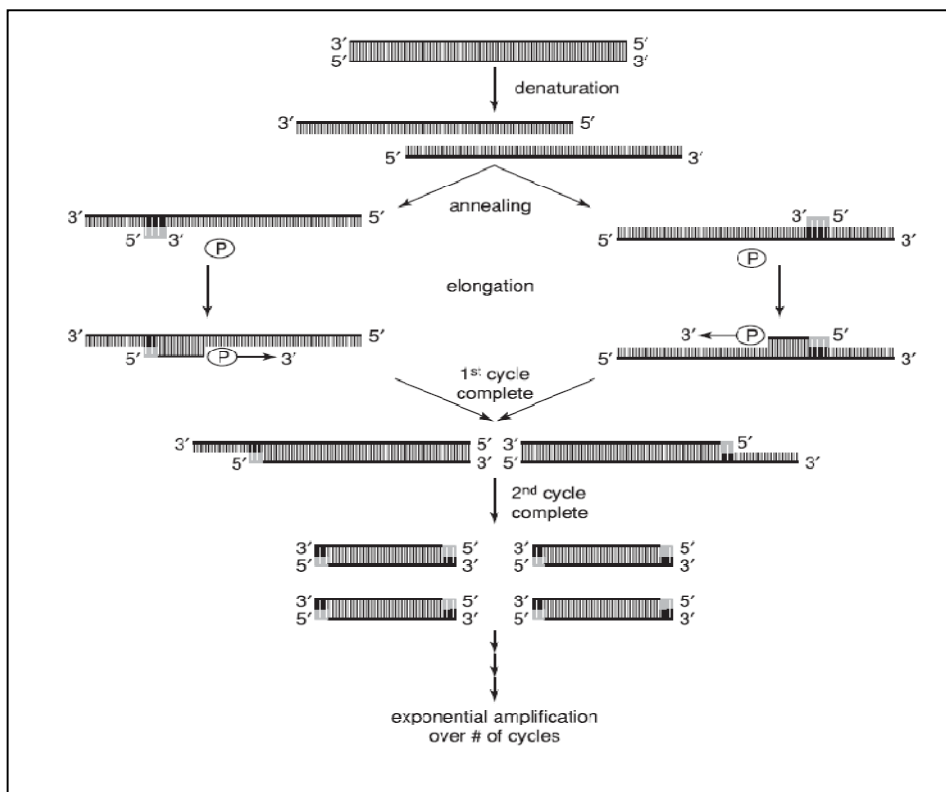


Fig.1. Schematic representation of PCR

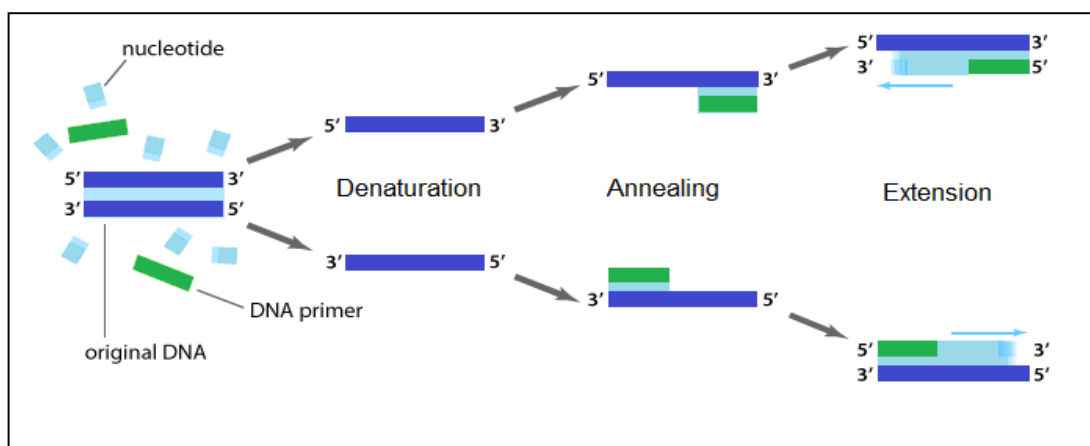


Fig 2. Illustration of steps in PCR—denaturation, annealing, extension, to amplify target sequence from a template DNA.

1.3 PCR protocol

Materials required

- a. 10% (w/v) bleach solution
- b. Master mix components (see Table)
- c. 5 U/ μ l DNA polymerase (e.g., *Taq*)
- d. 10 \times PCR buffer without MgCl₂ typically optimized for DNA polymerase of choice and provided by the manufacturer of the enzyme
- e. 25 mM MgCl₂
- g. 10 mM dNTPs (dATP, dTTP, dCTP, and dGTP)
- h. 10 μ M forward (upstream) and 10 μ M reverse (downstream) primer
- i. Molecular-biology-grade, sterile, nuclease-free ddH₂O
- j. DNA template
- k. Sterile, nuclease-free mineral oil (only necessary if thermal cycler does not have heated lid)
- l. Gel loading buffer/dye
- m. Agarose gel
- n. 100-bp PCR ladder
- o. Ethidium bromide or SYBR Green

Prepare reaction mixture

1. Wipe the work surface with 10% bleach solution.
2. Thaw PCR components on ice. Once all the components have completely thawed, vortex the reagents before using.
3. Prepare a master mix on ice according to the table except template and vortex it
4. Pipette out 49 μ l of the master mix into each thin-walled PCR tube (200 μ l), finally add 1 μ l template to each tube. Keep the tubes on ice.

Amplification conditions

Program the thermal cycler with the prevalidated PCR conditions done on the specific characteristics of the primers, template, and size of the amplicon.

Follow the PCR conditions as described mentioned in table

Amplicon analysis

1. Agarose or nondenaturing polyacrylamide is typically used to make a gel for PCR analysis. The concentration of the agarose gel will depend on the expected PCR product size i.e 0.5% to 2% agarose gels are adequate for many PCR products. It is recommended to use separate pipette tips for amplicons and aliquots of PCR reagents.
2. Ideally mix 5 to 10 μ l of amplicons with a gel loading dye (typically 1:1 to 1:6 PCR product:loading dye)
3. Load the mixture in agarose well, simultaneously run a PCR ladder of known sizes and electrophorese the mixture
4. Depending on the size of the amplicon, the concentration of the gel matrix, run the gel between 80 to 120 V for 20 to 45 min.
5. Stain the agarose gel with ethidium bromide, visualize on a UV transilluminator and capture the image under gel documentation unit
6. Analyze the PCR product with the DNA size ladder loaded on the gel to enable estimation of amplicon size.

1.4 Table 3. PCR Troubleshooting

Problem	Probable causes	suggestions
Little or no product amplification is observed	Incorrect Thermal cycling conditions	Consider using a prevalidated PCR reaction as a control to produce a specific known product
	Thermal cycling conditions are not optimal	Verify the optimal annealing temperature and check the initial hot-start activation step.
	Reagent may be omitted	Run a positive control to ensure that mastermix contain all the reagents
	Insufficient template	Amount of template has to be improved but not to exceed 1 μ g/100 ml reaction. Extra template is required for long PCR
	DNA template quality was poor	Isolate new DNA template by another method and store it under iced conditions.
	Mg ²⁺ concentration not optimal	Execute a Mg ²⁺ titration of about 0.5 mM increment
	Lack of sufficient enzyme activity	Increase the amount of enzyme in 0.5 U intervals; enzyme reagent not stored properly, exposed to many freeze/thaw cycles, or reagent

		expired
	No: of cycles are not adequate	Increase the number of cycles
	Primer sequence not optimal	Review considerations in primer design
Nonspecific products, background, or primer dimer formation	Excess DNA input was introduced in the reaction	Cut down the quantity of template DNA until background smearing gets disappeared
	Too many cycles	Lessen the total number of cycles until background gets disappeared
	High amounts of enzyme was added to reaction	Decrease the enzyme quantity in the reaction.
	Carryover contamination	Set a reaction with no DNA template control. If product or background is obvious in the control, reagents are contaminated with PCR products or templates. Replace all reagents with fresh ones and follow good PCR practices
	Annealing temperature is too low	Scale up the annealing temperature in 2 °C degree
	Too much primer was added to the reaction	Excess addition of primer may create primer-dimer or other nonspecific products. Titrate the amount of primers until primer-dimer or other nonspecific products are greatly reduced.

1.5. Table 4. PCR diagnosis of seafood bacterial pathogens

Table Primer sequence for seafood bacterial pathogens

Sl. No.	Pathogen	Target gene	Primer Sequence 5' to 3'	Amplicon size (bp)
1	<i>Escherichia coli</i>	<i>Uid</i>	CGC CGA TGC AGA TAT TCG GCT GTG ACG CAC AGT TCA TAG	604
2.	<i>Staphylococcus aureus</i>	16s rRNA	AACTCTGTTATTAGG GAAGAA CA CCA CCT TCC TCC GGT TTG TCA CC	759
3.	<i>Vibrio parahaemolyticus</i>	<i>Tlh</i>	CAT TAC TCC CGC TTG CTT CTG GCG AAC ATA GGT ATA GGT TTG GTT	590

1.5.1 PCR conditions for *Uid* gene- *Escherichia coli*

Cycle 1: 94°C for 5 min

Cycle 2:94°C for 30sec

60°C for 30sec

72°C for 45sec

Go to cycle 2 repeat 29 cycles

Cycle 3:72°C for 5 min

PCR conditions for 16s rRNA- *Staphylococcus aureus*

Cycle 1: 94°C for 3 min
Cycle 2:94°C for 1 min
55°C for 30sec
72°C for 1:30 min
Go to cycle 2 repeat 36 cycles
Cycle 3:72°C for 3.30 min

PCR conditions for *Tlh* gene- *Vibrio parahaemolyticus*

Cycle 1: 94°C for 3 min
Cycle 2:94°C for 1 min
58°C for 1min
72°C for 1 min
Go to cycle 2 repeat 29 cycles
Cycle 3:72°C for 3 min

References

Sambrook, J., Fritsch, E.F. and Maniatis, T., 1989. *Molecular cloning: a laboratory manual* (No. Ed. 2). Cold spring harbor laboratory press.

Kuslich, C. D., Chui, B., & Yamashiro, C. T., (2018). Overview of PCR. *Current Protocols Essential Laboratory Techniques*.

Molecular Diagnosis of Shrimp Diseases- A Training Manual, CIBA-TM Series 2016 No. 4

Applications of PCR vis-à-vis food microbiology and disease diagnosis:

The simplicity and the versatility of the PCR technique have revolutionized disease diagnosis and investigation. The rapid detection of human pathogens in food is critical for ensuring the safety of consumers, because contamination of food with pathogenic microorganisms is still one of the major problems of the food industry. Traditional methods to detect food-borne bacteria often rely on time-consuming growth in culture media (up to 4 days), followed by isolation, biochemical and serological identification. PCR-based assays are in general more specific, sensitive, and faster than conventional microbiological methods.

⇒ PCR methods have been developed to detect a wide range of organisms, whether they are present in foodstuff, the environment or biological or histological materials.

- ⇒ PCR looks directly for the bacteria / virus's unique DNA whereas serological tests look for indirect evidence that the microbe is present by searching for antibodies that the animal (host) has made against it.
- ⇒ Characterization and detection of infectious disease organisms have been revolutionized by PCR. PCR-based tests have allowed detection of disease organisms including the pathogens whose *in vitro* cultivation is difficult, time-consuming or unavailable.
- ⇒ PCR can detect bacteria that are in the viable but nonculturable (VBNC) state but still maintain their virulence potential.
- ⇒ PCR techniques are most useful for the detection of viruses for which cell culture and serological methods are laborious, expensive or unavailable. PCR is the method of choice for investigations on shrimp viral diseases, either endemic, emerging or exotic diseases.
- ⇒ PCR based methods help to evaluate antiviral treatments.
- ⇒ PCR methods help in ascertaining the distribution of the microbe in the host tissues thereby helping in determining microbial pathogenesis.
- ⇒ The spread of pathogenic microorganisms in human and animal populations can be monitored by PCR testing.
- ⇒ PCR aids in genetic testing, where a sample of DNA is analyzed for the presence of genetic disease mutations
- ⇒ The development of PCR-based fingerprinting protocols has seen widespread application in epidemiological investigations.
- ⇒ Genetic and physical chromosome maps have also been prepared, both in plants and animals, using PCR.

Types of PCR Assays

- ⇒ There are several variations to the standard PCR assay, which allow varied application of this technique.

2. Nested PCR (2 step PCR)

Nested PCR is also known as 2 step PCR because two separate amplifications are used using two pairs of PCR primers. The first pair of PCR primers (external primers) amplify a fragment similar to a standard PCR and yields a large amplicon. The larger fragment produced by the first round of PCR serves as template DNA for the second PCR. The second pair of primers called nested primers (that are internal to the first primer pair) bind to sequences within the initial product and produce a second smaller product (Fig. 1.).

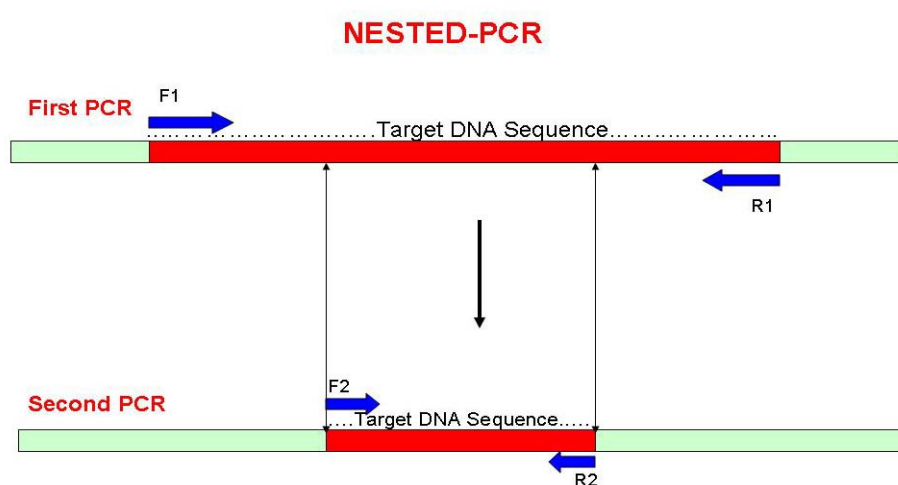


Fig.. Schematic representation of Nested PCR

F1 and R1 : External primers for First PCR, F2 and R2: Nested Primers for Second PCR

The advantage of two-stage amplifications of target DNA with nested pairs of primers increases the sensitivity and specificity of the PCR method. If the wrong PCR fragment was amplified in the first amplification (non-specific amplification), the probability that the region would be amplified by the second set of primers is very low. After the first round of PCR the non-specific products would not be complementary to the nested primers and hence unable to serve as template for further amplification. Thus, Nested PCR is a very specific PCR amplification. The need for verification of the PCR product by blotting, restriction digestion or sequencing is not necessary as the second set of primers also serves to verify the specificity of the first product.

Lo et al. (1996) developed highly specific nested primer sets for two-step PCR derived from the sequence of a cloned White Spot Syndrome Virus (WSSV) *Sal I* 1461bp DNA fragment for WSSV

diagnosis. The external primers 146F1 and 146R1, prime the amplification of a 1447bp fragment while the nested primers 146F2 and 146R2 prime the amplification of a 941bp fragment. The sensitivity of the two-step amplification was shown to be 10^3 to 10^4 times greater than the of one-step amplification alone. With two-step WSSV diagnostic PCR, they could detect 10 – 50 copies of the target DNA in a PCR solution. Due to its specificity and sensitivity, two-step WSSV diagnostic PCR should be useful both for identifying carriers in shrimp larvae, parental spawners and invertebrate populations which share the same habitat (Lo and Kou, 1998). Similarly, Lee et al., (1999) studied the effectiveness of two-stage Nested PCR for direct detection of *Vibrio vulnificus* in natural samples using external primers designed to amplify 704-bp and nested primers to amplify 222-bp fragments within the region of hemolysin gene, *vvhA*. The nested PCR amplification, coupled with direct extraction of template DNA, revealed improved sensitivity sufficient for detection of 1 to 10 CfU of *V. vulnificus* in 1 mL of seafood homogenates.

3. Semi-nested PCR

In the semi-nested PCR, only one of the second round primers is located within the amplified region and is used together with one of the first round primers (Fig. 2).

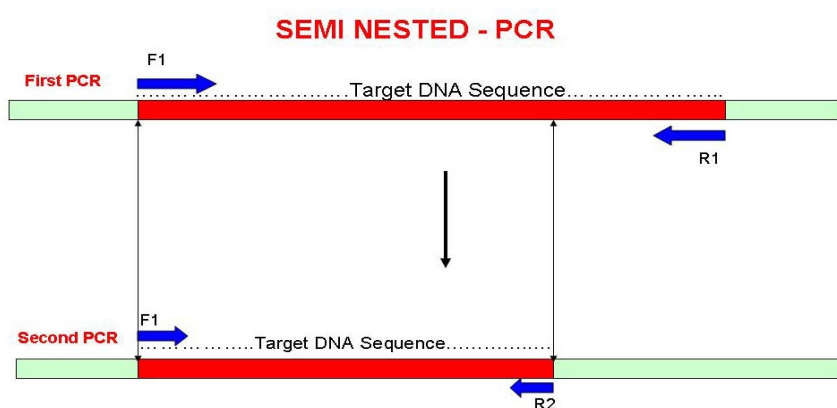


Fig. Schematic representation of Semi-Nested PCR, F1 and R1 : Primers for First PCR; F1 and R2: Primers for Second PCR

Saux et al (2002) applied seminested reverse transcription-PCR (RT-PCR) to viable but nonculturable (VBNC) populations of *V. vulnificus* and targeted the cytotoxin-hemolysin virulence gene *vvhA*. The two primers VV1 and VV2R delineate a 704-bp region within the open reading frame of the cytotoxin-hemolysin gene *vvhA* that is unique to *V. vulnificus*. The internal primer

VV3 is used in the seminested PCR in conjunction with VV2R, and generates a 604-bp fragment. Miyamoto et al. (1997) devised a seminested PCR assay for detection of *Legionella* species. For the first-step PCR, two oligonucleotides viz., LEG 225 and LEG 858 were used as amplimers enclosing a 654-bp fragment of the 16S rRNA gene. For the second-step PCR, two primers viz., LEG 448 and LEG 858 were used as another set of amplimers enclosing a 430-bp fragment of the 16S rRNA gene. The assay was found to be specific to legionellae, and the sensitivity was 1 fg of extracted *Legionella* DNA in laboratory examination.

4. Multiplex PCR

In multiplex PCR (mPCR), two or more primer pairs specific for different targets are included in the same amplification reaction. Multiplex PCR methods that can detect two or more pathogens in a single PCR assay have been used by several researchers. The target sequences for amplification were usually species specific regions of the pathogens or unique genes of each pathogen.

The major advantages of mPCR are

- ⇒ conservation of reagents and template
- ⇒ reduction in preparation and analysis time
- ⇒ identify multiple target sites in one assay

Multiplex-PCR methods require perfect optimization of the PCR conditions to ensure that one PCR reaction is not dominant over the other. The amplicons obtained in each reaction should also be of different sizes to enable their unambiguous visualization on agarose gel. The co-amplification of two or more products in a single reaction is dependent on the compatibility of the PCR primers used in the reaction. All the primers in the reaction must have similar melting temperature, so they anneal to and dissociate from complementary DNA sequences at approximately the same temperature; allowing each amplification to proceed at the selected temperature. Each amplification proceeds independently of the other. Once the conditions for a reaction have been tested, the reaction can be simply repeated.

Multiplex Reaction Conditions: Primer sequences should be designed so that their predicted hybridization kinetics are similar to those of other primers in the multiplex reaction. Primer annealing temperatures and concentrations may be calculated to some extent, but conditions will

almost certainly have to be refined empirically. The possibility of nonspecific priming and other artifacts is increased with each additional primer. Primer pairs that give a clean signal alone but produce artifact bands in multiplex may benefit from annealing at the highest possible temperature. If equimolar primer concentrations do not yield uniform amplification signals for all fragments, the concentration of some primer pairs can be reduced in relation to others. This is particularly important in samples where one target is more abundant than others. Mg^{2+} and dNTP requirements generally increase with the number of amplicons in the multiplex, but the concentrations must be optimized because each primer pair may have different requirements. Polymerase requirements generally increase with the size of the multiplex. Multiple sets of primers increase the possibility of primer complementarity at the 3' ends, leading to "primer-dimers." These artifacts deplete the reaction of dNTPs and primers and out compete the multiplex amplicons for polymerase. This effect can be reduced by titrating primer concentrations and cycling conditions.

mPCR assay finds application in disease monitoring, especially new, emerging and exotic diseases. Multiplexes indicate a particular pathogen among others, or distinguish species or strains of the same genus. Few of the methods targeting three or more bacteria are listed below.

Table : Multiplex PCR methods targeting food borne pathogens

Target Organisms	Target gene / sequence	Reference
<i>V.cholerae</i> , <i>Choleratoxigenic V.cholerae</i> , <i>V.alginolyticus</i> , <i>V.vulnificus</i> , <i>V.parahaemolyticus</i>	<i>sodB</i> for <i>V.cholerae</i> , <i>ctxAB</i> for <i>choleratoxigenic V.cholerae</i> , <i>gyrB</i> for <i>V.alginolyticus</i> , <i>hsp60</i> for <i>V. vulnificus</i> , <i>flaE</i> sequence for <i>V.parahaemolyticus</i>	Rao, B.M. (2010)

5. Duplex PCR

Duplex PCR is basically a multiplex PCR in which two primer pairs specific for two different targets are included in a single amplification reaction. The targets are usually two different genes or simply two specific sequences on the same organism. A duplex PCR targeting the genes *gyrB* and *tl* for specific identification of *V.parahaemolyticus* (Vongxay *et al.*, 2006) and *vh* fragment and

viuB for identification of *V.vulnificus*, (Panicker *et al.*, 2004) were reported. Rao (2010) developed a *V.cholerae*-duplex PCR method by utilizing *V.cholerae* species specific *sodB* primers and *ctxAB* genes specific primers. Cholera toxin production encoded by the *ctxAB* genes is the major factor in the pathogenesis of cholera and the presence of *ctxAB* is a prerequisite for full blown cholera disease (*Cholera gravis*) to occur. This method simultaneously detects *V.cholerae* isolates and differentiate *ctx* toxin producing *V.cholerae* strains. Non cholera toxin producing *V.cholerae* cultures yielded a single amplicon of 248bp whereas cholera toxin producing *V.cholerae* cultures yielded two amplicons viz., 248bp and 777bp (Fig. 3)

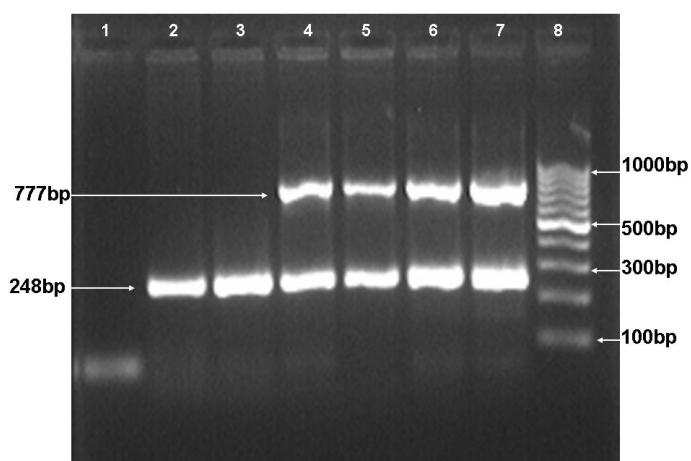


Fig. Duplex PCR for the simultaneous detection of *V.cholerae* and differentiation of cholera toxin producing *V.cholerae* isolates (Rao, 2010).

Lane 1, Negative control; Lane 2–3, Cholera toxin negative *V.cholerae* isolates from shrimp pond water; Lane 4-6, Cholera toxin positive *V.cholerae* isolates from shrimp pond water; Lane 7, *V.cholerae* (MTCC 3906); Lane 8, 100bp DNA ladder (Gene RulerTM, Fermentas)

6. RT-PCR (Reverse Transcription Polymerase Chain Reaction)

⇒ RT-PCR is based on the process of reverse transcription. Reverse transcription is the reverse transcriptase-mediated synthesis of single-stranded DNA (complementary DNA or cDNA) using single-stranded RNA as template. The cDNA product and the template

RNA have complementary sequence and can exist as RNA-DNA hybrid. The RNA in this hybrid state, is susceptible to degradation by RNase H.

- ⇒ RT-PCR is 2 step process. The First step of RT-PCR is referred to as the "first strand reaction". In the first-strand reaction, complementary DNA also termed cDNA, is made from the messenger RNA template of interest using oligo dT, dNTPs, and an RNA-dependent DNA polymerase, reverse transcriptase, through the process of reverse transcription. These components are combined in a reverse transcriptase buffer for 1 hour at 37°C. After reverse transcriptase reaction is complete, and the cDNA has been synthesized, RNaseH is added which digests the RNA away from the RNA-cDNA hybrid. After incubation with RNaseH, standard PCR is conducted using DNA primers specific for the sequence of interest and DNA polymerase. This second step is referred to as the "second strand reaction". Finally, the single stranded DNA becomes double stranded and is amplified, allowing the detection of RNA sequences.
- ⇒ Oligonucleotide poly-dTs act similar to primers and bind to the 3' polyA sequence located at the 3' untranslated region, which are present in most mRNAs. The two commonly used reverse transcriptases are AMV-RT (Avian myeloblastosis virus reverse transcriptase) and MMLV-RT (Moloney murine leukemia virus reverse transcriptase). For RNA templates with high G+C content or complex secondary structure, the high-temperature reverse transcriptase activity of thermostable *rTth* DNA Polymerase is effective. Recombinant *Thermus thermophilus* (*rTth*) DNA Polymerase, thermostable recombinant DNA polymerase, designed for amplification of DNA or RNA targets.
- ⇒ RT-PCR is useful for the detection of RNA viral diseases in shrimp viz., Yellow Head Virus, Taura Syndrome Virus and food borne pathogens viz., enteroviruses. RT-PCR needs to be performed with special care as RNA is highly unstable because the ribose sugar carries hydroxyl groups in both 2' and 3' positions while RNase is highly stable because of the intrachain disulphide bonds.

7. Real-Time PCR (kinetic PCR, qPCR)

- ⇒ Real-Time PCR is a quantitative PCR that has completely revolutionized the detection of RNA and DNA. As the name suggests, one can visually see the progress of the reaction in "real time". Traditional PCR methods use agarose gels for detection of PCR amplification at end of the reaction, while Real-Time PCR collects data in the exponential phase. Monitoring the amount of fluorescence emitted during the PCR reaction indicates the amount of PCR amplification that occurs during each PCR cycle. An increase in the reporter fluorescent signal in Real-Time PCR is directly proportional to the number of amplicons generated. Thus, as a PCR product accumulates, fluorescence increases. The starting copy number of the target is determined by monitoring when PCR product was first detected; the higher the starting copy number of the target the sooner a significant increase in the fluorescence is detected. The fluorescence data is continuously analysed by the Real Time PCR machine which displays the results eliminating the need for post-PCR processing (Fig. 4a).
- ⇒ Real Time PCR amplification is performed in PCR strips or 96 well plates. A laser or filter beam of specific wavelength is directed to each of the sample wells and the fluorescence emission data for each sample well is collected once every few seconds as the PCR products are generated. The fluorescence is spectrally analyzed and the results are displayed on the computer monitor, eliminating the need for agarose gel electrophoresis.
- ⇒ Four basic tools are used in Real-Time PCR viz., DNA-binding dyes, Molecular beacons, hybridization probes and hydrolysis probes. The simplest method uses fluorescent dyes that bind specifically to double stranded DNA. DNA-binding dye based Real-Time PCR method involves detection of the binding of a fluorescent dye (SYBR Green) to DNA. SYBR Green I is currently the most frequently used intercalating dye in real-time PCR. It has a 100 times higher binding affinity than ethidium bromide, and the fluorescence of bound dye is more than 1000-fold higher than that of free dye. SYBR Green dye is non-sequence specific fluorescent intercalating agent. It does not bind to single stranded DNA (ssDNA). SYBR green is a fluorogenic minor groove binding dye that exhibits little

fluorescence when in solution but emits a strong fluorescent signal upon binding to double-stranded DNA (dsDNA). During elongation, increasing amounts of the dye bind to the nascent double-stranded DNA. When monitored in real-time, this results in an increase in the fluorescence signal that can be observed during the polymerization step and that falls off when the DNA is denatured. Consequently fluorescent measurements at the end of the elongation step of every PCR cycle are performed to monitor the increasing amount of amplified DNA. Fluorescence values are recorded during every cycle and represent the amount of product amplified to that point in the amplification reaction. The more the template present at the beginning of the amplification reaction, the fewer number of cycles it takes to reach a point in which the fluorescence signal is first recorded as statistically significant above background. This point is defined as C_T (cycle threshold) and will always occur during the exponential phase of amplification. This method obviates the need for target specific fluorescent probes but its specificity is determined entirely by its primers. Non-specific amplifications require follow-up assays (melting point curve or dissociation analysis) for amplicon identification. Normally SYBR green is used in singleplex reactions, however when coupled with melting point analysis, it can be used for multiplex reactions. Melt curve analysis ensures specificity of the amplified PCR products. Melt curve analysis should show a single melting maximum for each sample indicating specific amplification without primer-dimer (Fig 4b).

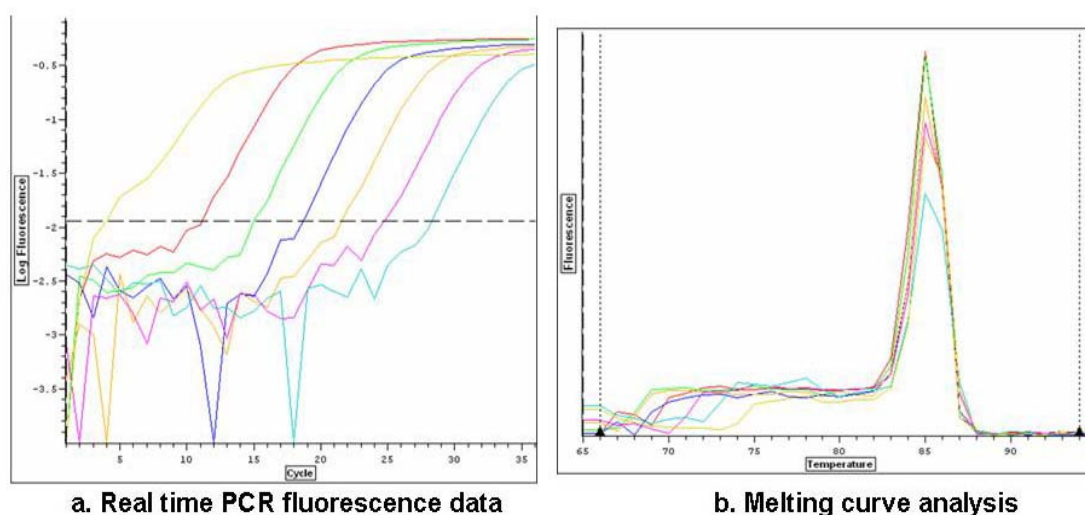


Fig. Real Time PCR data analysis

- ⇒ The other chemistries used in Real Time PCR viz., Molecular Beacons (Stratagene), Hybridization Probe and Hydrolysis probe (The Taqman assay of Perkin Elmer – Applied Biosystem) rely on the specific hybridization of fluorescence labeled probe to the correct amplicon.
- ⇒ Real-Time PCR methods were developed and used for the detection of human pathogens. The advantages of Real Time PCR in microbial detection are its higher sensitivity, faster result and quantify the microbial load.

Table: Real Time PCR methods for detection of pathogenic vibrios.

Organism	Real Time PCR method	Reference
<i>V.cholerae</i>	SYBR Green	Gubala (2006)
<i>V.cholerae</i>	TaqMan PCR	Lyon (2001)
<i>V.cholerae</i>	Molecular beacon	Gubala and Proll (2006), Fykse et al (2007)
<i>V.parahaemolyticus</i>	SYBR Green	Blackstone et al (2003)
<i>V.vulnificus</i>	SYBR Green	Panicker et al (2004a)
<i>V.alginolyticus</i>	SYBR Green	Zhou et al (2007)

PCR fingerprinting:

- ⇒ PCR based methods are employed to study the genetic heterogeneity of different bacterial species. The commonly used methods are RAPD-PCR, AFLP-PCR, RS-PCR, REP-PCR and ERIC-PCR. These approaches are all based upon identification of polymorphic loci. Polymorphic loci are regions or points in the genome that may vary from one strain (bacteria / viral) to another. Strain specific arrays of DNA fragments (fingerprints) are generated by PCR amplification.

8. RAPD-PCR (Randomly amplified polymorphic DNA-PCR)

- ⇒ RAPD-PCR technique (Welsh and McClelland, 1990; Williams *et al.*, 1990) is based on amplification of random DNA segments with single primers of arbitrary nucleotide sequence, i.e., random amplified polymorphic DNA-PCR, arbitrary primer PCR. This

method is also called as Arbitrarily Primed PCR. A short oligonucleotide primer is used to produce multiple and random sets of amplified DNA fragments. The multiple products resulting from RAPD analysis are then separated according to size by conventional agarose gel electrophoresis and the DNA banding patterns of different isolates can then be compared. This technique was used for subtyping diverse bacterial species. RAPD with short primers may be particularly vulnerable to artefactual variations due to slight differences in PCR conditions (Ellsworth *et al.*, 1993; Wong *et al.*, 1999) and is complicated by variations in band intensity and the lack of reproducibility of certain minor bands (Samore *et al.*, 1996). Therefore, PCR typing methods using specific primers designed on the basis of the repeated and conserved sequences in bacteria and more stringent annealing conditions display more promising fingerprints than RAPD analysis.

9. PCR-RFLP (Restriction Fragment Length Polymorphism)

⇒ In this method, DNA is extracted and standard PCR is performed with primers. The amplified PCR product is digested with restriction enzymes. The amplified fragments are then electrophoresed on agarose gels. The discriminatory power of RFLP analysis of amplicons obtained from mitochondrial DNA has been utilized for animal species identification (Meyer *et al.*, 1995; Ram *et al.*, 1996, Cocolin *et al.*, 2000). Analysis of RFLP variation was an important tool in genome mapping, localization of genetic disease genes, determination of risk for a disease, genetic fingerprinting, and paternity testing.

10. AFLP-PCR (Amplified Fragment Length Polymorphism PCR)

⇒ AFLP PCR is a highly sensitive PCR-based method for detecting polymorphisms in DNA (Vos *et al.*, 1995). AFLP is a combination of RAPD and RFLP. AFLP can be also used for genotyping individuals for a large number of loci using a minimal number of PCR reactions. Initially, cellular DNA is cut with one or more restriction enzymes. Typically this involves a combination of two restriction enzymes: a 4 base cutter and a 6 base cutter. Ligation of linkers (restriction half-site specific adaptors) to all restriction fragments is performed. PCR is performed using primers which match the linkers and restriction site specific sequences which produces many fragments, some of which may vary in length from individual to individual.

11. RS-PCR (Ribosomal Gene Spacer sequence-PCR)

⇒ Spacer regions within the 16S and 23S genes in prokaryotic rRNA genetic loci exhibit significant length and sequence polymorphisms in different species and are flanked by highly conserved sequences (Jensen *et al.*, 1993). Multiple copies of these loci occur in bacteria (Srivastava *et al.*, 1990). Therefore, amplification using primers designed on the basis of these flanking sequences will generate polymorphic fingerprints which can be used to distinguish bacterial strains at the species and subspecies levels (Al-Saif *et al.*, 1998; Bidet *et al.*, 2000). RS-PCR has been applied to typing of species from many genera, including *Listeria*, *Staphylococcus*, and *Salmonella* (Jensen *et al.*, 1993, Lagatolla *et al.*, 1996).

12. REP-PCR (Repetitive Extragenic Palindrome sequence-PCR)

⇒ The REP-PCR method is based on the presence of 38-bp REPs in *Enterobacteriaceae* and other bacteria and has been applied to many species (Marshall *et al.*, 1999; Rodriguez *et al.*, 1995; Stern *et al.*, 1984; Stubbs *et al.*, 1999).

13. ERIC-PCR (Enterobacterial Repetitive Intergenic Consensus sequence PCR)

⇒ ERIC sequences are 126 bp long and appear to be restricted to transcribed regions of the genome, either in intergenic regions of polycistronic operons or in untranslated regions upstream or downstream of open reading frames. ERIC sequences are novel and highly conserved at the nucleotide sequence level, but their chromosomal locations differ between species (Hulton *et al.*, 1991). The ERIC sequence in *V.cholerae* has been identified and is located near the hemolysin gene, apparently “hitchhiking” with the hemolysin gene. It is possible, by ERIC-PCR, to generate a characteristic genomic fingerprint for given bacterial species, including *V.mimicus*, *V.vulnificus*, *V.paraahaemolyticus*, *V.campbelli*, *V.mediterranei*, *V.alginolyticus*, *Escherichia coli*, *Shigella sonnei*, *Shigella dysenteriae*, *Shigella boydii*, *Shigella flexneri*, and *Bacillus subtilis*, which can be used to distinguish patterns of particular strains (Rivera *et al.*, 1995, Versalovic *et al.*, 1991).

⇒ Rao and Surendran (2010) carried out PCR fingerprinting of *V. cholerae* isolated from black tiger (*Penaeus monodon*) shrimp farms using RS-, REP- and ERIC-PCR methods. They reported that RS-PCR (Fig.5a) yielded fewer bands (maximum 4) when compared to REP-PCR (maximum 10 bands) and ERIC-PCR (maximum 12 bands) (Fig. 5b). 100% similarity between *V.cholerae* isolates obtained from shrimp aquaculture was noticed only in RS-PCR.

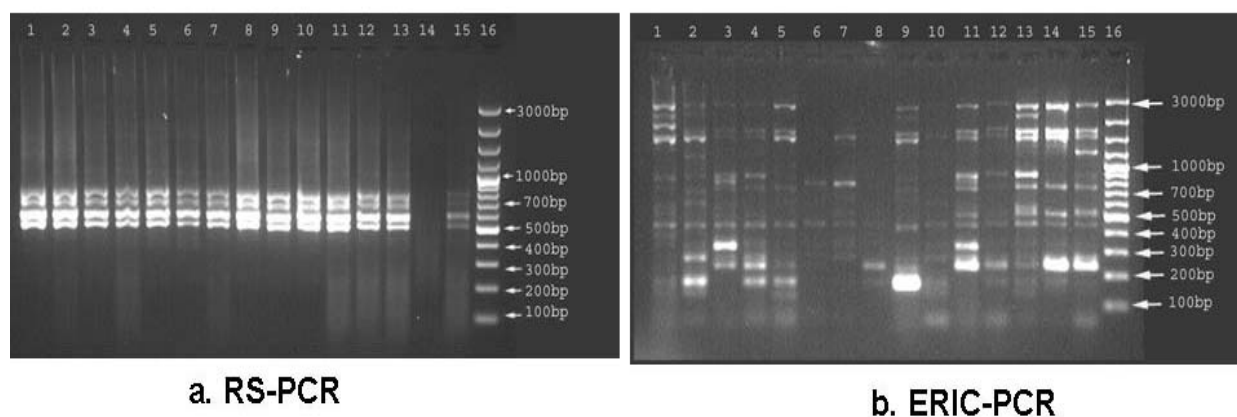


Fig. PCR fingerprinting of *V. cholerae* isolated from black tiger (*Penaeus monodon*) shrimp farms using RS-PCR and ERIC-PCR

14. Degenerate PCR

This is a technique in which nucleotide sequence of the primer is based on the sequence of the encoded protein. The genetic code is said to be degenerate because some amino acids are encoded by more than one codon. From the amino acid sequence of a protein, it is possible to design PCR primers based on all the principal codon sequences for each amino acid. Degenerate PCR is used to amplify conserved sequences of a gene or genes from the genome of an organism and to get the nucleotide sequence after having sequenced some amino acids from a protein of interest. Sequences amplified this way can then be sequenced to confirm that the sequence is correct. They can then be used as

probes to fish out the gene of interest from a genomic library (prokaryotic) or a cDNA library (eukaryotic). This method is also used when there has been a successful isolation of a protein of interest. The terminals of this protein (or some amino acids) were then sequenced. The amino acid sequence can then be used to design degenerate primers. The PCR product can then be sequenced.

15. HOT-Start PCR

- ⇒ Hot-Start PCR is a method for obtaining cleaner PCR products. All the constituents of PCR mix viz., template DNA, PCR buffer, primers, dNTPs are mixed together and held at a temperature above the threshold of non-specific binding of primer to template DNA. Just before cycling, the missing component i.e., Taq polymerase is added to allow the reaction to take place at higher temperature. The amplified bands tend to be cleaner as the primers don't have a chance to anneal non-specifically. Hot-start PCR can also be performed by creating a physical barrier (wax layer) between the template and primers. Mixing of the components occurs at high temperature only when the wax melts. Another way is by using AmpliTaq Gold® DNA polymerase, which is an inactive enzyme that requires heat activation to regenerate its polymerase activity. The necessity for thermal activation provides highly specific PCR conditions resulting in lower background and increased yield of specific product. A 10 minute - 95 °C pre-PCR incubation step, provides an efficient Hot-Start PCR delivering the appropriate amount of enzyme activity throughout the amplification process.

- ⇒ PCR assay and its many variations provide sensitive, specific and swift detection and quantification of DNA/RNA making them indispensable tools for state-of-the-art diagnostics of important human and animal pathogens. PCR continues to have major impact on disease diagnosis and risk analysis of foods. Miniaturization of these PCR assays and making them commercially available at reasonable costs is the challenge ahead.

14. EU directives pertaining to Food and Feed

1	Food Law	(EC) 178/2002
2	General Hygiene Criteria Reg.	(EC) 852/2004
3	Specific Hygiene Criteria Reg.	(EC) 853/2004
4	Official control of Feed and Food General Reg.	(EC) 882/2004
5	Official Control of Feed and Food of Animal origin Reg.	(EC) 854/2004
6	Water Quality Directive	98/83/EC
7	Microbial and Chemical criteria Reg.	2073/2006, (EC) 1881/2006, (EC) 2074/2005
8	Food Additives Reg.	(EC) 1333/2008
9	Labelling Reg.	(EC) 1169/2011
10	Requirements to sampling and testing Reg.	(EC) 333/2007 and (EC) 589/2014
11	Minimum required performance limits (MRPLs) for certain residues in food of animal origin (Antibiotics)	2003/181/EC
12	sampling methods and the methods of analysis for the official control of the levels of benzo(a)pyrene in foodstuffs	2005/10/EC
13	Amending Regulation (EC) No 466/2001 as regards heavy metals	(EC) No 78/2005
14	setting of minimum required performance limits (MRPLs) for certain residues in food of animal origin (malachite green and leucomalachite green)	2004/25/EC
15	Fixing the total volatile basic nitrogen (TVB-N) limit values for certain categories of fishery products	95/149/EC
16	Food additives other than colours and sweeteners	95/2/EC
17	Feed hygiene Reg.	(EC) 183/2005
18	Veterinary Medicines Dir.	82/2001/EC
19	Aquatic Animal Health and disease control Dir.	88/2006/EC
20	Animal by-product rules Reg.	(EC) 1069/2009 and Reg. (EC) 142/2011