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Polymerase Chain Reaction Technique for Microbial and Environmental investigations

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Introduction

The PCR can amplify specific sequences of DNA and has been developed as a recent specific and sensitive technique that can be used for direct detection of microorganisms in aquatic (Bej et al, 1990), food or dairy products (Golsteyn Thomas et al, 1991), and terrestrial environments (Bruce et al, 1992; Picard et al, 1992). PCR can also be used to detect microorganisms environments that are difficult to culture in vitro and to determine the fate of particular organisms or genetically engineered microorganisms (Steffan et Atlas, 1988). This technique can also be used to study complex natural environments from which only a small percentage of the microflora can be isolated in vitro (Staub et al, 1994; Torsvik et al, 1990; Tsai et al, 1993; Ward et al, 1990) such as enteric viruses. In fact, enteric virus transmission due to the consumption of feacally-contaminated potable water, foods and shellfish is a significant public health concern (Richards, 1985; Sobsey, 1987). While the fecal coliform groups of bacteria are accepted as the criterion for the sanitary quality universally of potable water, foods and shellfish, the scientific consensus is that these indicators do not reflect the occurrence of enteric viruses in the marine environment (Gerba, 1988).

In the absence of indicator systems, investigators have attempted the effective detection of viruses from marine sediments, shellfish and their harvesting direct waters. These techniques have relied on cumbersome methods of virus extraction and subsequent detection based on virus infectivity for susceptible, live laboratory hosts (Sobsey, 1987). These virus concentration and detection steps are expensive, time-consuming, technically difficult, and lacking in sensitivity. They are further limited by the need for large sample sizes to detect the low virus levels that still pose a health risk and the lack of susceptible hosts for the more epidemiologically important viruses, such as hepatitis A virus (HAV) and the Norwalk- type viruses (Jaykus et al, 1994; Sobsey, 1987). Reverse transcriptase (RT) PCR is a rapid, economical, sensitive, and specific approach (Chapman et al, 1990; Rotbart, 1990), which has been used recently for the amplification of nucleic acid sequences for the HAV (Jansen et al., 1990), rotaviruses (Gouvea et al, 1990), and Norwalk enteroviruses virus (De Leon et al., 1990) in clinical fecal specimens for which expected viral contamination levels are high. However, there is a need to develop methodology designed specifically to detect the lower levels of viral contamination typically expected in environmental samples.

Principle of the PCR

The purpose of a PCR (Polymerase Chain Reaction) is to make a huge number of copies of a gene. This is necessary to have enough starting template for sequencing. ***The cycling reactions:*** There are three major steps in a PCR, which are repeated for 30 or 40

cycles. This is done on an automated cycler, which can heat and cool the tubes with the reaction mixture in a very short time.

Denaturation at 94°C: During the denaturation, the double strand melts open to single stranded DNA, all enzymatic reactions stop (for example: the extension from a previous cycle).
Annealing at 54°C: The primers are jiggling around, caused by the Brownian motion. Hydrogen bonds are constantly formed and broken between the single stranded primer and the single stranded template. The more stable bonds last a little bit longer (primers that fit exactly) and on that little piece of double stranded DNA (template and primer), the polymerase can attach and starts copying the template.

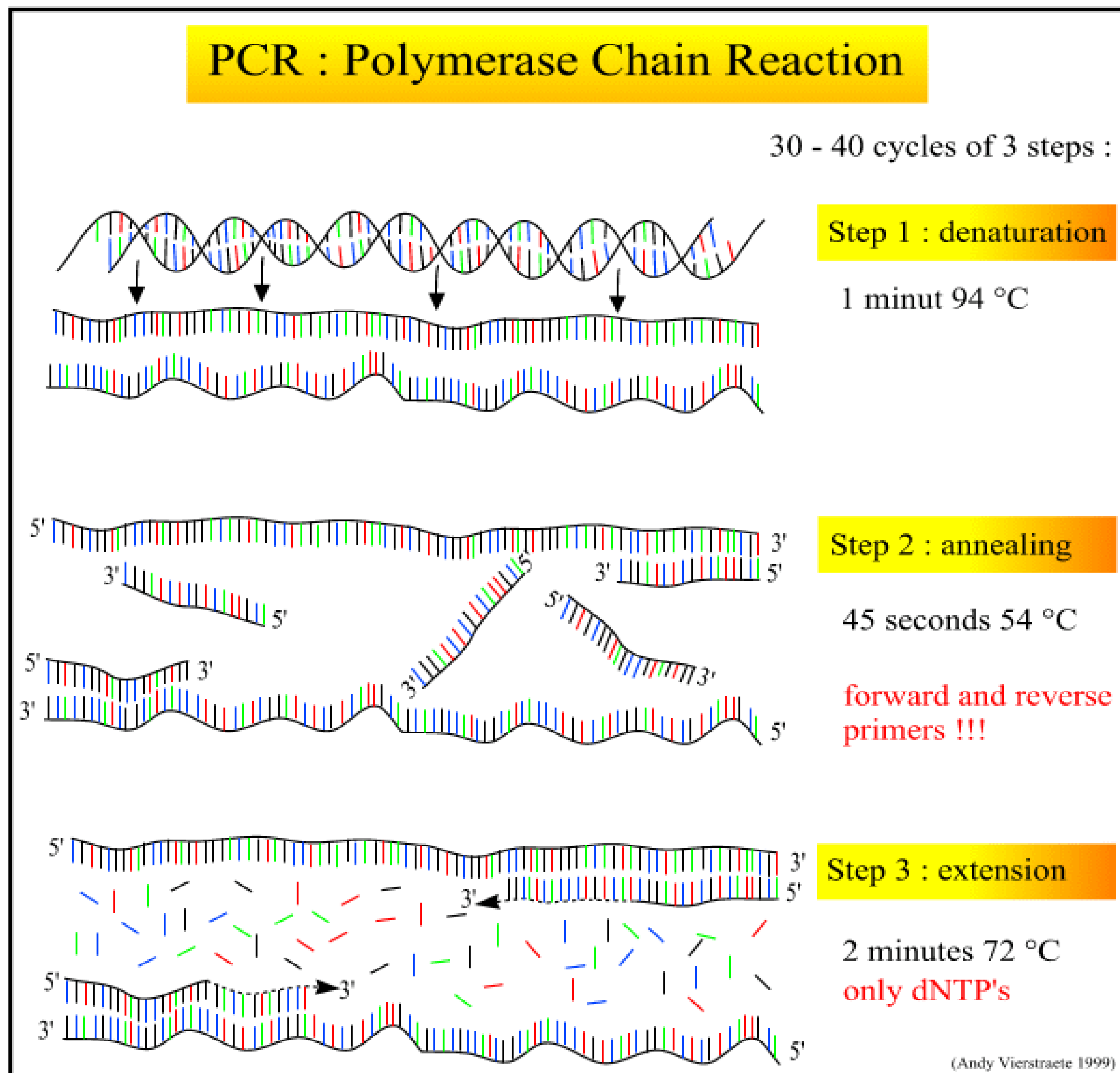

Fig. 1. The different steps in PCR

1. Once there are a few bases built in, the hydrogen bond is so strong between the template and the primer that it does not break anymore (Fig. 1).

2. Extension at 72°C: This is the ideal working temperature for the polymerase. The primers, where there are a few bases built in, have a stronger attraction to the template, created by hydrogen bonds, than the forces breaking these attractions. Primers that are on positions with no exact match get loose again (because of the higher temperature) and don't give an extension of the fragment.

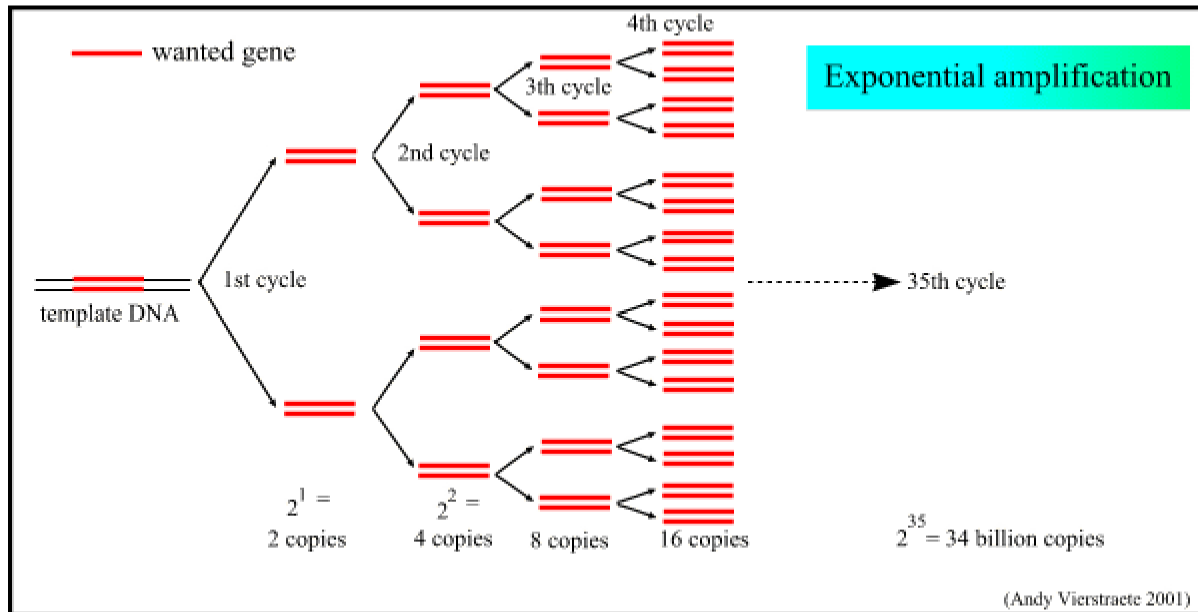

Fig. 2. The exponential amplification of the gene in PCR.

The bases (complementary to the template) are coupled to the primer on the 3' side (the polymerase adds dNTP's from 5' to 3', reading the template from 3' to 5' side; bases are added complementary to the template). Because both strands are copied during PCR, there is an **exponential** increase of the number of copies of the gene. Suppose there is only one copy of the wanted gene before the cycling starts, after one cycle, there will be 2 copies, after two cycles, there will be 4 copies, three cycles will result in 8 copies and so on (Fig. 2).

Objectives

The goal of this research financed by the Japan international Cooperation Agency (JICA) was to develop procedures to detect intact enteric viruses from superficial sediments of coastal lagoons of Bizerte and to identify dominant microbial species and analyze the phylogenetic relationship between these strains.

Preliminary investigations

In this first step of study, we have used the ribosomal operon (a classic molecular marker) commonly used to summarize genetic relationships and to identify strains rapidly. Moreover, ribosomal operon analysis can differentiate between the genomic lineages of a single species, as in the case of *Pediococcus acidilactici* strains that produce, or not, pediocin AcH/PA1 and in bacteriocinogenic baccilli respect to sensitive strains. Of all the different regions of the ribosomal operon, the 16S rDNA and the internal transcribed spacers (ITS)

between 16S and 23S rDNA are frequently used as molecular markers to identify microbial species and analyze the phylogenetic relationship between strains.

Material and methods

To extract DNA 1.5 ml of an overnight culture in LB broth was pelletted and the cells washed three times with physiological saline. Total DNA was extracted from the washed cells by SDS-proteinase K treatment. Amplification of the 16S rDNA and the ITS was performed using the already described primers (Table 1). PCR was performed with a *BIOMETRTA T-Personal* automated thermocycler with 0.2-ml thin walled PCR tubes. Reactions were carried out in 50 ul-volumes containing 5 ul of 10 X PCR buffer (supplied with *Taq* DNA polymerase, Appligene), 2.0 mM $MgCl_2$, 50 pM of each oligonucleotide primer (Table 1), 0.2 mM each deoxynucleotide triphosphate (dATP, dCTP, dTTP and dGTP), 1 U of *Taq* DNA polymerase (Appligene) and 50 ng of extracted DNA. Amplification of the 16S rDNA and the ITS was attempted with 30 cycles at 95°C for 30s, 55°C for 45s and 72°C for 45s, followed by a final extension step at 72°C for 7 mn.

Table 1. Sequences and positions of the used primers.

Primer	Target Sequence	Position	Sequence 5'---3' [a]	Expected size
S-D-Bact-0008-a-S-20	ADNr 16S	8-28	AgAgTTTgATCCTggCTCAg	1486
S-D-Bact-1495-a-A-20	ADNr 16S	1474-1494	CTACggCTACCTTgTTACgA	
S-D-Bact-1494-a-S-20	ITS 16S-23S	1494ADNr 16S	gTCgTAACAAggTAgCCgTA	Pattern
S-D-Bact-0035-a-A-15	ITS 16S-23S	35 ADNr 23S	CAAggCATCCACCgT	

For each strain the PCR-products were analyzed individually by electrophoresis. The ITS-PCR and the 16S amplifications patterns were electrophoresed on standard 2% agarose gels in 1× Tris-Acetate-EDTA buffer (50X is 2 M Tris base, 57.1 ml Acetic Acid, 0.05 M EDTA-Na_2, pH 7.9) and stained for 30 min in a 0.5 mg/l solution of ethidium bromide.

Results

Preliminary results were summarized in Figure 3. Example of ITS-PCR fingerprinting patterns of Bacillus strains resolved by 2% agarose gel electrophoresis, showing the migration of some amplified products. Lanes M, 50 bp ladder. Lanes 1-9, *B. cereus* strains 256, 282, 582, 846, 7700, 7702, Cepanzo, 4229, Davis TE702. The 100 bp and 500 bp bands of the ladder are indicated.

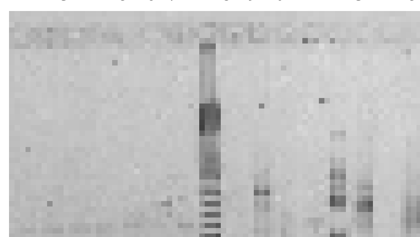

Fig. 3. ITS-PCR patterns

The rDNA-PCR technique can be used in strains identification (16S-ARDRA) and phylogenetic relationship investigations. Subsequent analysis can be performed such us sequencing in order to confirm identification. To enhance the efficiency of the technique we can also use the "GelDoc 2000" which is a powerful instrument that permits image capture in real time. With its software, image acquired can be rapidly optimized, annotated, analyzed and printed.

Perspectives

We programmed in the two next years 3 types of investigations

1. Direct detection of enteric viruses (Enteroviruses) in superficial sediments of the lagoon of Bizerte. These organisms are very difficult to culture in vitro and their transmission due to the consumption of contaminated water and shellfish is a significant public health concern.
2. Direct detection of microorganisms usually used as a microbial tracer in complex aquatic and terrestrial environments (such as compost, soils amended with organic residues, leachates, sediments, wastewater infiltration-percolation, etc.). Microorganisms implicated in this study are especially Bacillus, Actinomycetes, Pseudomonas and Salmonella.
3. Use of PCR investigation to determine the fate of particular organisms or genetically engineered microorganisms such as iron bacteria, sulfito-reducer bacteria, nitrifier bacteria etc.

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