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Standardization and Optimization of a Long Distance PCR Assay for *Foot-and-Mouth Disease Virus*

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Taq DNA polymerase with different concentrations of other thermostable DNA polymerases with 3' to 5' exonuclease activity (PFU and Vent) was used in the amplification of large fragments (2 Kb, 3 CD genes) of *foot and mouth disease virus* (FMDV). Such long distance polymerase chain reaction (LD-PCR) was conducted to reduce mismatch extension rates and improve epidemiological cloning and sequencing studies. When enzyme mixtures in the reaction were optimized, best results were obtained with Tth and PFU polymerases at concentrations of 2.50-0.40, 2.50-0.50 and 2.50-0.60 units, respectively.

INTRODUCTION

Over the last few years, there has been a revolution in the use of molecular epidemiology to study the spread of FMD episodes among many parts of the world. The ability of FMDV genome to accommodate considerable amounts of mutations during outbreaks has made the genome of this pathogen unamenable for reproducible nucleic acid sequence comparisons, tracking studies, sequencing and cloning (Mason *et al.*, 2003). Recently, however, the addition of a low level of a proofreading enzyme to the non-proofreading polymerase in the PCR reaction mixture was found to be more efficient in synthesizing full-length products and correcting the mismatch errors that are introduced during the process (Barnes, 1994). Use of LD-PCR had also overcome some shortcomings of the regular PCR. Not only did it speed up cloning or sequencing of genetic chains of interest, but it also enabled PCR to bridge large gaps of unknown or variable sequences between primers (Nobile *et al.*, 1997 and Kelley *et al.*, 1997). In fact, it has been shown that long PCR had preserved the distribution of variants of poliovirus (Chumakov 1996) and greatly facilitated the construction of infections clones or infectious cDNAs for HBV (Gunther *et al.*, 1995), *tick borne encephalitis virus* (Tellier *et al.*, 1996) and *hepatitis A virus* (Tellier *et al.*, 1997). Since many viral sequences may be toxic to *Escherichia coli*, leading to selection bias in cloning procedures, large amplicons were found to be readily sequenceable and transcribable (Forns *et al.*, 1997). The purpose of this study was to develop and evaluate a long PCR procedure for foot and mouth disease virus (FMDV) in order to circumvent foreseeable problems in its cloning and sequencing.

MATERIALS AND METHODS

Reverse transcription: Since RNA cannot be amplified in a thermocycler unless converted to complementary DNA (cDNA),

components of the reaction consisted of 20 µl, DNTP (4 µl/1mM), 2 µl template specific primers (200 ng, Table 1), 1 µl FMDV RNA (genome Aga 93), 0.5 µl (20 units) RNasin (Promega) and 0.6 µl reverse transcriptase (about 50 units).

Table (1) primers Sequences of 3 CD genes of FMDV

Primer (1)	'5 G/GAA/TTC/CAT/ATG/AG T/GGT/GCC/CCA/CCG/AC C/GAC/TT '3
Primer (2)	'5 GCG/CAT/ATG/AAT/TCG/ CGT/CAC/CGC/ACA/CGC ' CGT/TCA '3

This master mix was placed in a thermocycler programmed as follows: (1) 23°C for 10 minutes, (2) 42°C for 30 minutes, (3) 98°C for 7 minutes, and (4) 25°C for hold.

To amplify cDNA by long PCR, the following ingredients were included in the reaction mixture: 1 µl cDNA, 5 µl 10x Tth buffer, 6 µl 1mM DNTPs, primers 1.5µl + 1.5 µl (about 150 ng. each), 2.5 units Tth polymerase, and 30.5 µl DEPC H_2O (free from RNase, DNAse, and proteinase). The mixture was covered with about 30 µl of mineral oil. However, different enzymes in different concentration were employed to optimize the reaction, as described in Table (2).

Thermocycler program used consisted of the following phases: (1) 95°C for 3 minutes, (2) 80°C hot start for 3 minutes with polymerase enzymes, (3) Annealing for 45 sec at 55°C, (4) extension at 72°C for 1.45 minutes, denaturation at 94°C for 45 sec, (6) repetition of steps 2, 4, 5 for 35 cycles, (7) extension for 10 minutes at 72°C, (8) hold at 25°C.

RESULTS AND DISCUSSION

Our optimization experiments showed that large templates were produced by PCR using DNA taq polymerase in addition to different concentrations of other thermostable DNA polymerases that had 3' to 5' exonuclease activity (PFU and Vent). The first long RT PCR was performed using Tth polymerase alone and showed extra bands as shown in Fig 1, lanes 1, 2, 3, 4 and 5.

Table (2): Different concentrations of polymerase enzymes in the reaction mixture of long RT PCR.

Experiment No	Type of polymerase	Concentration of each, respectively
1-	Tth DNA Polymerase	2.5 units + 00
2-	Tth DNA polymerase + PFU polymerase	2.5 units + 0.6 unit
3-	Tth DNA polymerase + PFU polymerase	2.5 units + 0.5 unit
4-	Tth DNA polymerase + PFU polymerase	2.5 units + 0.4 unit
5-	Tth DNA polymerase + Vent polymerase	2.5 units + 0.6 unit
6-	Tth DNA polymerase + Vent polymerase	2.5 units + 0.5 unit
7-	Tth DNA polymerase + Vent polymerase	2.5 units + 0.4 unit

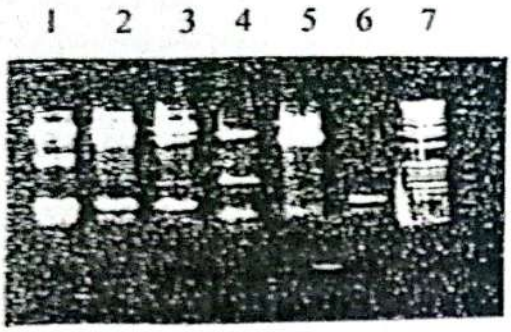
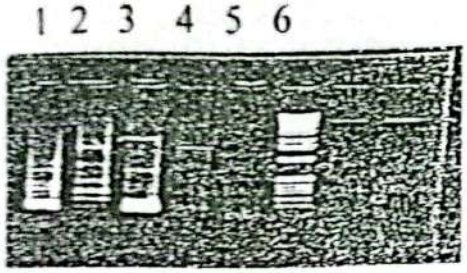


Figure (1). Use of Tth Polymerase

When long RT PCR was performed using Vent and Tth polymerases (Table 2), different results were obtained (Figure 2, lanes 1, 2 and 3).



Figure(2) . Use of Tth Polymerase along with Vent polymerase.

Using a different concentration of PFU polymerase with Tth polymerase (Table 2), a sharp clear band was observed (Figure 3, lane 2). By comparing the use of different polymerase enzymes in different concentrations, best results were obtained when Tth and PFU mix were included at a ratio of 5 to 1, respectively (Figure 3, lane 2).

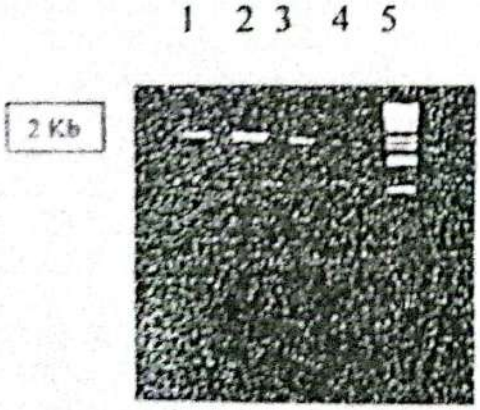


Fig (3) Use of Tth polymerase with different concentrations of PFU polymerase

Polymerase chain reaction (PCR) has become an essential tool for biological research and laboratory diagnostic applications. The technique was especially important in FMD diagnosis due to the rapid spread and devastating economical effects of the disease. Other attempts were made to use this technique for tracking FMD virus infections and comparison among strains. Until recently, reliable and sensitive amplification of large DNA (or cDNA) templates (several Kb) was associated with high error (mismatch) rates, leading to inefficient elongation (Fig. 1) by taq polymerase alone (Barnes, 1994). However, use of mixtures of non-proofreading and proofreading DNA polymerases was shown to allow the amplification of longer templates (long distance PCR or LD-PCR) than is possible with single enzyme formulations (Barnes, 1994).

The greater the fidelity of the long PCR enzymes (Tth and PFU polymerases), the better were the results (Cline *et al.*, 1996). In this study, optimization of enzyme mix concentrations, proper buffers and thermocycling conditions were very important to obtain clear and sharp bands as shown in Fig (3). This observation has been also supported by Cheng *et al.*, (1994).

In general, long PCR worked better with rapid temperature transitions, smaller reaction volumes, use of a superficial layer of mineral oil and thin walled tubes, in agreement with Barnes (1994) and Cheng *et al.*, (1996). LD-PCR has been applied with great success to viral genomes of *hepatitis C virus* (HCV; Rispeter *et al.*, 1997), *bovine torovirus virus* (Duckmanton *et al.*, 1998), and the full length of potato virus genome (Fakhfakh *et al.*, 1996). LD-PCR had

also been used to amplify the complete human mitochondrial genome (Li, y-y *et al.*, 1995), large DNA fragments to screen for determining deletions in human genetic diseases (Bochmann. *et al.*, 1996) and gene fusions in cancer patients (Waggott *et al.*, 1995).

Currently, there are several commercially available long PCR kits that are made from different enzymes and require somewhat different protocols. Since most are expensive, it may be recommended to use our protocol as a model for similar viral genomes, particularly in developing countries.

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