P—9

DETECTION OF Qβ COLIPHAGE BY USING THE POLYMERASE CHAIN REACTION

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1. Introduction

RNA-F-specific coliphages were considered by many researchers to serve as useful models for the behaviour of human enteric viruses in wastewater treatment process. The verification of the fate of RNA-F-specific coliphages in wastewater ecology requires a tracking method for isolation and detection to determine theirs growth, survival and dessemination in the environment. An innovative method of **in vitro** DNA amplification by mean of the polymerase chain reaction (PCR) recently developed, seems to have an outstanding potential to offer the possibility of the fulfillment of this requirement. Phage group III of RNA-F-specific coliphages was reasonably found to be introduced from the human source (Furuse K., 1987). Therefore, Qβ coliphage which belongs to group III phage was firstly investigated as a case study by using PCR as a tool for the detection. Here, the preliminary study of this developed method on the ability of two synthesized primers to amplify Qβ RNA and sensitivity of PCR protocol are reported.

2. Materials and Method

Qβ Culture and Extraction Qβ coliphage was propagated by using E. coli K-12 F^+, A/λ as a host cell. The filtrated Qβ lysate was uncontaminated from liberated materials of the lysed host cells by rinsing with sterilized TM buffer (50 ml of 1M Tris-HCl, pH 7.5 and 2g of $MgSO_4.7H_2O$ in 1 liter of H_2O) in an autoclaved ultrafiltration module. Qβ RNA of a purified Qβ coliphage was extracted by using phenol-chloroform method, whereas, Qβ RNA extraction of the filtrated Qβ lysate was also performed by directly heating at 90°C for 5 min. These Qβ RNAs were utilized for testing the ability of Qβ RNA amplification and sensitivity of the PCR protocol.

Primers Figure 1. illustrates the location and specific sequences of the primer pair that we use in this protocol. Primer 1 is antisense to genomic Qβ RNA and primer 2 is sense. These genomic regions of the two primers are 100% sequence conservation in the 3' end terminal of Qβ and ST phages that have been reported (Inokuchi, etal, 1982). The two synthesized primers were purchased from Takara Shuzo, Company, Ltd.

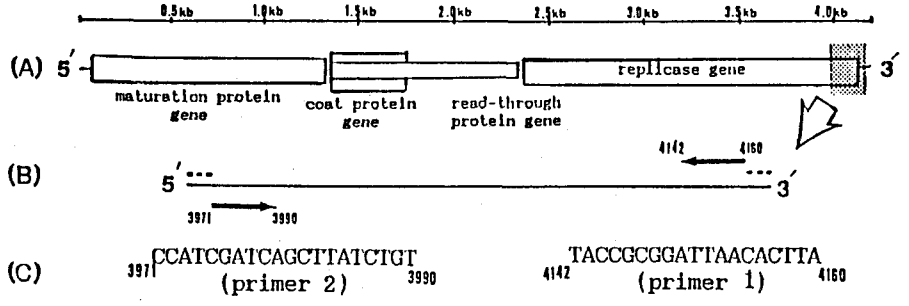

Fig 1. (A) Structure of Qβ phage genomes. (B) Enlargement of the 3' end terminal region to present the location of the two primers. (C) Base sequences of two primers.

Reverse Transcription and PCR Protocol The Qβ RNAs from the extraction above was heated at 90°C for 5 min and chilled on ice. The 3 μL of heat treated Qβ RNA was added to the 1 μL of 40 units of RNasin, 2.5 μL of 5X reverse transcription buffer[250mM Tris-HCl, pH 8.3, 15mM $MgCl_2$, 350mM KCl, 50mM DTT], and 1 μL each of 10 mM dATP, dCTP, dGTP, and dTTP. One μL of the primer 1 (10 p mol/μL) and 1 μL of avian myeloblastosis virus reverse transcriptase (5 units/μL) were added last. The 12.5 μL mixture was overlayed with 80-100 μL of mineral oil and incubated at 37°C, for 60 min to test the ability of Qβ RNA amplification and for 90 min to test the sensitivity of PCR protocol.

The amplification reaction was made up in a total mixture volume of 40 μL by direct adding the following reagents to the reverse transcription mixture: 8 μL of sterilized distilled water, 4 μL of 10X PCR buffer [560 mM KCl, 100 mM Tris-HCl, pH 8.3, 15 mM $MgCl_2$ and 0.1% gelatin (w/v)], 6.5 μL of dNTP mixture (2mM each), 4 μL of primer 1 (10 pmol/ μL), 4 μL of primer 2 (10 pmol/μL), and 1 μL of Ampli Taq DNA polymerase (0.5 unit/μL). PCR was performed by using DNA Termal Cycler. A reaction cycle that worked for testing the ability of Qβ RNA amplification is a 5-minute denaturation at 95°C and following by 15 cycles of annealing (50°C, 2min), primer extension (72°C, 2min) and denaturation (95°C, 2min). The final step is in a 9-minute primer extension at 72°C. While the experiment of testing the sensitivity of PCR protocol, a thermal cycle profile is (1) denaturing for 30 sec at 95°C, (2) cooling over 1 min to 50°C, (3) annealing primers for 30 sec at 50°C, (4) heating over 30 sec to 72°C, (5) extending the primers for 30 sec at 72°C, (6) heating over 1 min to 95°C. The reaction of PCR was performed 25 cycles. After the end of 25 cycles, 3 μL of the amplified product was added to a fresh reaction mix and further amplified for 25 cycles, resulting in a dual PCR amplification

Detection of PCR Products The PCR products were detected by using agarose gel electrophoresis. A 6.4 μL sample of the PCR product was loaded along with the loading dye onto 4% NuSieve 3:1 agarose gel and run for 50 min in 1X TAE buffer (0.04 M Tris-acetate and 0.001 M EDTA) at 16.7 volts/cm. On completion of the electrophoresis, the gel was stained with ethidium bromide and photographed during UV-light exposure. The reference size of ØX 174 digested by Hae III was used as DNA marker for identification the PCR products.

3. Results and Discussion

Amplification of Qβ RNA The PCR technique was developed to amplify RNA in the 3' end terminal region of Qβ genome. To test whether the 2 synthesized primers would be able to amplify Qβ RNA, PCR was initially performed by using 84 and 90 ng of Qβ RNA extracted from phenol-chloroform method and RNA of 1.29 X 10^8 PFU of Qβ coliphage which extracted by direct heating as the starting template. The amplification was performed for 15 cycles. As shown in Fig.2, amplification of Qβ RNA from both of two methods extraction were occurred, as evidence by the presence of a DNA fragment with size near 194 bp. The expected size of the amplification DNA in the gene of Qβ RNA was 190 bp. From this result, it can be said that two synthesized primers are successful to amplify Qβ RNA. Moreover, direct heating Qβ coliphage at 90°C for few minute is capability to destroy Qβ capsid and allows the ability of Qβ RNA amplification .

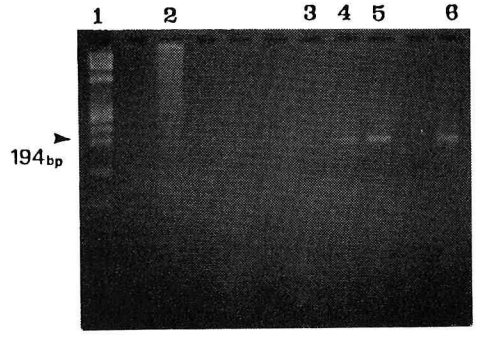

Fig. 2 Agarose gel electrophoresis showing the result of amplification of Qβ RNA. Lane 1 was the size marker of ØX 174/ Hae III digested. Lane 2 was unamplified Qβ RNA. Lane 3 was negative control. Lane 4 and 5 were the amplified Qβ RNA applied by using the amounts of 84 and 90 ng respectively. Lane 6 was amplified Qβ RNA applied by using the amounts of 1.29 X 10^8 PFU of Qβ coliphage which RNA extracted by direct heating.

Determination of Sensitivity for the PCR Technique To determine sensitivity of the PCR technique and the detection of the PCR product on agarose gel electrophoresis, first-stage and second-stage amplification of a set 10-fold serial dilution of the Qβ RNAs were conducted. The amounts of the Qβ RNA in the reactions were ranged from 90 ng to 90 ag and 8.1 x10^8 PFU to 8.1 x 10^{-3} PFU of Qβ RNAs extracted from phenol-chloroform method and direct heating method, respectively. In the first-stage amplification, the DNA fragment with 190 bp in size was amplified in the reactions containing only the amounts of Qβ RNAs ranging from 90 ng to 90 pg and 8.1 x 10^8 to 8.1 x 10^6 PFU. (data not present here) After the second-stage amplification, up to 0.9 pg of Qβ RNA and 8.1 x 10^4 PFU of Qβ coliphage yielded sufficient amplified products for visualization on an ethidium bromide-stained agarose gel. These results are demonstrated in Fig. 3 and 4.

From our study of the extracted Qβ RNA recovery by using phenol-chloroform method, the amount of 0.9 pg of Qβ RNA is corresponded to 3.6 x 10^3 PFU of Qβ coliphage. So, the sensitivity of detection limit is in contrast to the detection limit obtained from extracted Qβ RNA by using direct heating. This difference in sensitivity can be affected from the quality of the Qβ RNA to amplification. The impurity of Qβ capsids may sufficiently inhibit the reverse transcription reaction and the polymerase chain reaction.

4. Conclusion

In conclusion, the developed PCR method to detect Qβ coliphage can be simply done by using two synthesized primers of 5'..ATTCACAATTAGGCGCCAT..3' and 5'.. CCATCGATCAGCTTATCTGT..3'. The detectable amplification of Qβ RNA is 0.9 pg and/or about 10^4 PFU of Qβ coliphage, when its RNA extracted by phenol-chloroform method and direct heat. This is the limited sensitivity of Qβ RNA amplification by this dual PCR method and detection by ethidium bromide-stained agarose gel.

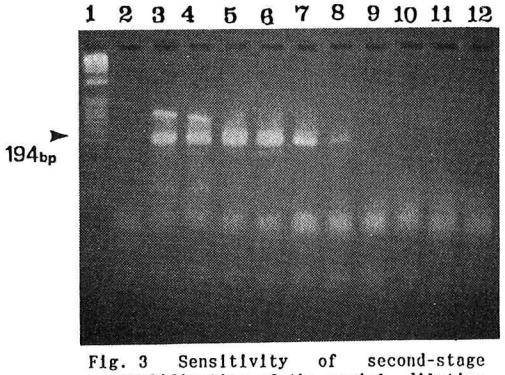

Fig. 3 Sensitivity of second-stage amplification of the serial dilution of Qβ RNA which extracted from phenol -chloroform method: detection by agarose gel electrophoresis. Lane 1: marker DNA, Lane 2: negative control. Lane 3 to 12 were loaded with 6.4 μL each of the PCR products. Lane 3: 90 ng, Lane 4: 9 ng, Lane 5: 0.9 ng, Lane 6: 90 pg, Lane 7: 9 pg, Lane 8: 0.9 pg, Lane 9: 90 fg, Lane 10: 9 fg, Lane 11: 0.9 fg, and Lane 12: 90 ag.

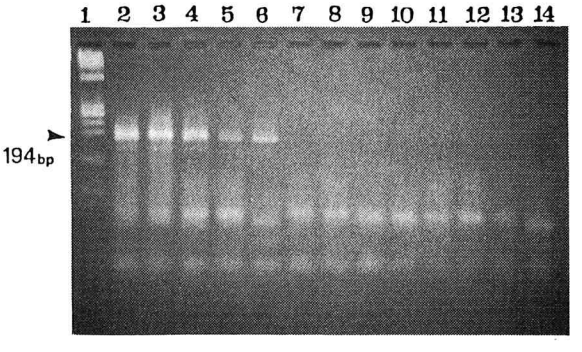

Fig. 4 Agarose gel electrophoresis showing the second-stage amplification of the serially diluted Qβ RNA which extracted by direct heat. Lane 1 was the marker DNA. Lane 2 to 13 were loaded with 6.4 μL each of the PCR products . Lane 2: 8.1 x 10^8PFU, Lane 3: 8.1 x 10^7 PFU, Lane 4: 8.1 x 10^6 PFU, Lane 5: 8.1 x 10^5 PFU, Lane 6: 8.1 x 10^4 PFU, Lane 7: 8.1 x 10^3 PFU, Lane 8: 8.1 x 10^2 PFU, Lane 9: 8.1 x 10^1 PFU, Lane 10: 8.1 x 10^0 PFU, Lane 11: 8.1 x 10^{-1} PFU, Lane 12: 8.1 x 10^{-2} PFU, Lane 13: 8.1 x 10^{-3} PFU, and Lane 14: negative control.

Reference

1.Furuse K. (1987) " Distribution of coliphage in the environment: General consideration" from Phage Ecology, Goyal M., Gerba C.P., and Bitton G. (eds), Wiley & Sons, Inc. New York, 87-124.

2.Inokuchi Y., Hirashima A., and Watanabe I., (1982), Comparison of the nucleotide sequences at the 3'-terminal region of RNA coliphages, J. Mol. Biol., 158:711-730.