Establishment of a Real-Time PCR Analysis System to detect Enterovirus infections

Introduction

There have been several enteroviris outbreaks in Taiwan since the summer of 1998. Though these outbreaks have been controlled, there have been some serious infections of children and even deaths. Two laboratory methods are traditionally used to confirm diagnosis, the cell culture infectivity[1], and a molecular biological diagnosis method such as PCR[2]. A new method, real-time PCR, has been developed to directly detect enterovirus in specimens. This method combines the *Tag-Man* technique and the *ABI Prism*^{TM} 7900 real-time sequencing detection system. The method is highly sensitive, specific, as well as time and manpower-saving. The designed primers are specific for the non-coding region of the 5' section (a highly conserved region). One-step RT-PCR[3] was carried out to amplify to about 145 bps sections. By using the dual fluorescent labeled DNA probe and the AmpliTaq DNA's 5'→3' nucleolytic activity, specific PCR products can be detected and compared with standard of known quantity to estimate indirectly the quantity of the virus. To more precisely quantify the amount of enterovirus in specimens, the present

study used Mahoney type 1 polivirus RNA as standard and molecular cloning of the amplified PCR products from polivirus RNA into the pGEM-T Easy Vector. The dynamic range of this assay encompassed at least 7 orders of magnitude (10^1 - 10^7). It is useful in the mass screening of specimens. Any enteroviral titer within 10^1 - 10^7 in specimens can be detected immediately after real-time PCR reaction; the conventional way of detecting the presence of PCR products after PCR reaction is not necessary.

Materials and Method

Primers/probes

The primers and probes used in the present study were developed by the primer Express soft. They were positioned at the highly conserved region of the enterovirus 5' UTR section[4]. The forward primer EV-F was 5'–CCCCTGAATGCGGCTAATC–3'(position: 450-468), the reverse primer EV-R: 5'-GATTGTCACCATAAGCAGC-3' (position: 580-596), and the probe EV-Prove: 5'-FAM-CGGAACCGACTACTTTGGGTGTCCGT-TAMRA-3[4,5].

The pGEM-T Easy vector

It was a linear vector carrying on both ends 3'-T nucleotide and Amp^r gene. It could perform rapid ligation with 3'-A overhang to each end of the PCR product.

Establishing enterovirus standard

Using Mahoney type 1 polivirus full genome as template and EV-F/EV-R as primers, 145 bps gene sections were amplified by the RT-PCR method. The PCR products were attached into the pGEM-T Easy Vector (Promega) by using T4 ligase of the molecular biological genetic engineering method. The constructed vector was transfected into the *E. coli* of JM109. The ampicillin

resistance gene served as a selection marker for screening by ampicillin. The screened colonies were incubated at 37 ℃ for enrichment, DNA was extracted and tested for O.D. value, and diluted to 2-2x10^6 copies/ul.

Extraction of RNA

RNA purification was performed by using QIAGEN's QIAmp Viral RNA kit. 140 ul of specimen was collected, 560 ul of buffer AVL was added and placed at room temperature for 10 minutes; a further 560 ul of pure alcohol was added for complete vortexing. The above mix was placed through an AIAmp spin column, washed twice with the buffer AW; RNA was dissolved with 80 ℃ Rnase-free water. The virus RNA so prepared was used for the reverse transcription polymerase chain reaction.

RT-PCR Quantification

ABI 7900 was used for analysis. 5 ul of RNA was added to Qiagen one-step RT-PCR reaction mix. Concentration of the primer was 400 nM; concentration of the fluorescent labeled probe was 200 nM. The RT was produced at 48 ℃ for 30 minutes. AmpliTaq DNA polymerase was then activated at 95 ℃ for 10 minutes and PCR performed for 40 cycles for denaturing at 95 ℃ for 15 seconds, and annealing-extension at 60 ℃ for one minute. The fluorescent signal was collected during the step of annealing-extension. Analysis was made by the ABI Prism SDS soft.

Reverse Transcription Polymerase Chain Reaction (RT-PCR):

(1) Primer: 012-ATGTAYGTICCICCIGGIGG (2875-2894)
040-ATGTAYRTICCICCIMCIGGIGC (2905-2924)
011-GCICCIGAYTGITGICCRAA (3311-3292)

(2) Reverse transcription reaction: to 10 ul of virus RNA 75 mM KCl, 50 mM

Tris-HCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATCG dNTP mixture, 38 U/ul Rnasin, and 50 pmole antisense primer was added: R-RT probe, placed at 70 ℃ for 10 minutes, and a further 100 units of MuLV-reverse transcriptases was then added and placed at 37 ℃ for 90 minutes.

(3) Polymerase chain reaction (PCR): The cDNA collected from the reverse transcription reaction was used for PCR. To the cDNA was added 50 mM KCl, 10 mM Trais-HCl, 1.5 mM $MgCl_2$, 0.1% Triton-X 100, 1 mM ATCG dNTP mixture, and primer: 012/011 or 040/011 50 pmole each[6,7]. To the mixture was added further 5 units of Taq polymerase (Promega), placed at 94 ℃ for denaturing for 3 minutes, and reactions performed 35 times at 94 ℃ for 1 minute, primer temperature (Tm, varied from each primer) 1 minute, at 72 ℃ for 2 minutes, and finally at 72 ℃ for 15 minutes.

Culturing of virus and quantification

Specimens of either quantified or non-quantified virus were cultured with RD cells in 96 well plates. The medium was DMEM with 10% of fetal calf serum . To each well was added 50 μl of cell growth medium (except to the control well) and 100 μl of cell suspension fluid ($5x10^4$ cells), placed at 36 ℃, CO_2 culture box, and cytopathic effect was observed on the 4-5th days and 7^{th} day, and counted for TCID50 and amount of virus.

Results

Standard Curve of Enterovirus and Ct (Cycle Threshold) Value

The present study extracted the vectors containing fragments of highly conserved 5' UTR gene section of the enterovirus with Qiagen DNA extraction kit, measured for the O.D. 260 value; their weight-volume concentration (ug/ml)

was calculated first, and then volume-mole value (mole/ul), diluted 10 times to $2x10^6$ copies/ul), and analyzed with real-time 7900. The fluorescent values that were over threshold measured at different concentrations differed from the PCR amplification cycles. At a higher concentration, the measured fluorescent values would be higher than the threshold with fewer PCR amplification cycles; whereas under lower concentration, the results were reversed (see Figure 1). The Ct values were decided by the fluorescent values (ΔRn) of the standards of different concentrations and PCR amplification cycles .For example, when concentration was 10^7 copies, the Ct value was about 15. The Ct value increased by the decline of copies at 2-3 per decline of 1 log of copies. The smallest copies were 10, the Ct value was 38, and r^2 value>0.990.

The present study used ABI 7900 for RT-PCR for qualitative and quantitative analyses. Detection of fluorescent emission was made after the extension step of PCR by vertical laser stimulation. In quantitative analysis, the standard curve played a very important role in that all specimens to be tested had the quantity of virus estimated by the standard curve. The standard curve should, therefore, be carefully constructed at recommended values of slope 3.3, Y-Intercept 40, correlation 1.0[(3)]. The Ct value and quantity of the standard curve analyzed by the SDS soft are shown in Figure 2. The R2 value of the present study was 0.9988.

Clinical Testing of Specimens

The primers of enterovirus designed in the present study were in the highly conversed 5' UTR gene section. They could be used for the detection of all enteroviruses such as poliovirus, enterovirus 68-71, Echo virus, and Coxsackievirus[(8)]. Currently, the method is used in laboratories for the

detection of enterovirus 71, Eoho virus, and Coxsakievirus (data not shown).

Qualitative Analysis

Seven known types of enteroviruses including enterovirus 71, Coxsakievirus A2, A4, A6 and A16, and Eoho virus types 4 and 6, were selected for PCR qualitative analysis. 012/040/011 was used as primer for the RT-PCR reaction , the confirmation was done by electrophroresis and gel EtBr staining. Findings: no 436 bp of DNA amplification was found in the Eoho virus type 4 by conventional RT-PCR analysis; whereas in other enteroviruses, 436 bp sections were noted. However, by qualitative analysis using ABI 7900, the result showed that fluorescent emission (Ct values) of different intensities could be noted in each specimen, indicating that the seven specimens all contained enteroviruses. The real-time PCR could be used for qualitative analysis (see Figure 3).

Quantitative Analysis

The clinically isolated and cultured enterovirus 71, tested by TCID50 and calculated for copies number by the number of enteroviurs in each ml, and diluted 10 times after quantification. The diluted viruses were cultured and observed for their CPE and PCR analysis was performed. It was found that only a concentration range of 1,000 copies/ml could be detected by real-time 7900 for one-step RT-PCR analysis. The value detected was not much different from the actual number of diluted viruses[4]; the difference was smaller than 1 log (see Table). CPE could be observed from $3x10^8$ to $3x10^2$ copies/ml (see Table 1).

Discussion

Developing real-time PCR techniques for the detection and analysis of clinical specimens has become a trend. Due to their high sensitivity and

specificity, they can be used for the early detection of the types of pathogenic agents in specimens[9] to minimize time required for testing. Therefore, they are useful in prescribing appropriate medicine for treatment and in increasing the survival rate of patients. They can also provide disease control workers with prompt and accurate information on diseases for timely and effective control measures to prevent their spread.. To improve the sensitivity and specificity of real-time PCR, the design of the primers is most important in order to differentiate different types of pathogenic agents. In the designing of primers, highly specific gene regions are required. The Primer Express soft developed by the ABiosystem can meet the needs of researchers for the designing of primers and probes. The primers and probes used by the present study were designed, according to research findings of other countries, by the highly-conserved 5' UTR section of enterovirus[4]. The section is a highly-conserved section of all enteroviruses and is at the same time a high specific area different from other pathogenic agents. It can be used for the screening of all enteroviruses, though not for typing.

The system was used by the laboratory for the analysis of all known types of enteroviruses including enterovirus 71, Coxsakievirus A2, A4. A6 and A16, Eoho virus types 4, 6 and 8[7]. Fluorescent signals were noticed in all specimens, indicating that this system could be used to detect enteroviruses of different types, and that the primers designed were in the highly conserved regions of enterovirus genes. A region different from other types of enteroviruses would have to be used for the detection of different viruses such as EV71 to distinguish their differences.

In the quantitative analysis, the present study used enterovirus 71. Viruses of known concentration were diluted and analyzed quantitatively by this system to assess the accuracy of quantification. The difference, as can

be noted, between the findings through laboratory tests and the actual number of viruses was smaller than an acceptable 1 log. However, the system could test only concentrations higher than 1,000 copies/ml. The reasons for this being, that the system used primers to amplify DNA sections of enterovirus; extraction of nucleic acid would be required at the beginning of the study. The present study used the QIAmp Viral RNA kit of the QIAGEN for RNA extraction and purification to extract only 140 ul of nucleic acid. In the final elution, 60 ul AVE buffer was used. In the analysis with PCR 7900, only 5 ul of RNA was used as template for the reaction. In the final calculation of the amount of viruses, the test finding should be multiplied by the diluted value of 85.7 to represent the actual amount of viruses in 1 ml specimen. If 1,000 copies/ml was divided by 85.7, the amount for 11 copies/ml could be obtained, suggesting that if the concentrations of the PCR 7900 analysis were lower than 10 copies/ml, viruses were unlikely to be detected. The amount lost in the process of nucleic acid extraction should also be considered. The findings thus obtained were acceptable. To improve the sensitivity of real-time PCR, a higher concentration of specimens could be considered. For instance, when 1 ml of specimens was concentrated to 100 ul, sensitivity could be improved 10 times.

In the PCR quantification, a small amount of fluorescent singals could be noted in the none-temple control (NTC) well. The reasons could be that the instability of the probes resulting in hydrolysis made the 5' section release reporter dye FAM . In the dilution of probes, it would be advisable to keep them in TE buffer and kept separately to reduce length of time of freezing and un-freezing, thus to reducing the instability of probes. When three consecutive fluorescent singals appear, it can be decided that the detected singals are due to the amplification of DNA sections and not to

the hydrolysis of probes [8,10].

In addition to the primers and probes already designed for enteroviruses, efforts are being made to develop them for other viral pathogenic agents such as HIV-1, influenza A and B, HAV and EVB. We hope that they can be used soon in the routine testing of the Center for better and more rapid analysis.

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References

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Table 1. Quantitative Analysis of EV71 Specimens of Known Concentrations by Real-Time PCR, and Qualitative Analysis of Sensitivity by RT-PCR and Virus Culture CPE

Sample (Copies/ml)	Result of Real-time PCR (No. of Copies/ ml)	RT-PCR	Culture CPE
3×10^{8}	3.3×10^{8}	(+)	(+)
3×10^{7}	3.0×10^{7}	(+)	(+)
3×10^{6}	2.9×10^{6}	(+)	(+)
3×10^{5}	2.7×10^{5}	(-)	(+)
3×10^{4}	2.1×10^{4}	(-)	(+)
3×10^{3}	2.2×10^{3}	(-)	(+)
3×10^{2}	undetectable	(-)	(+)
3×10^{1}	undetectable	(-)	(-)

Figure 1. Analysis of Real-Time PCR Standard Curve for Enterovirus by ABI7900. The Figure is an amplification plot. The vertical axis is the value of fluorescence detected; and the cross axis is the cycles of PCR amplifications.

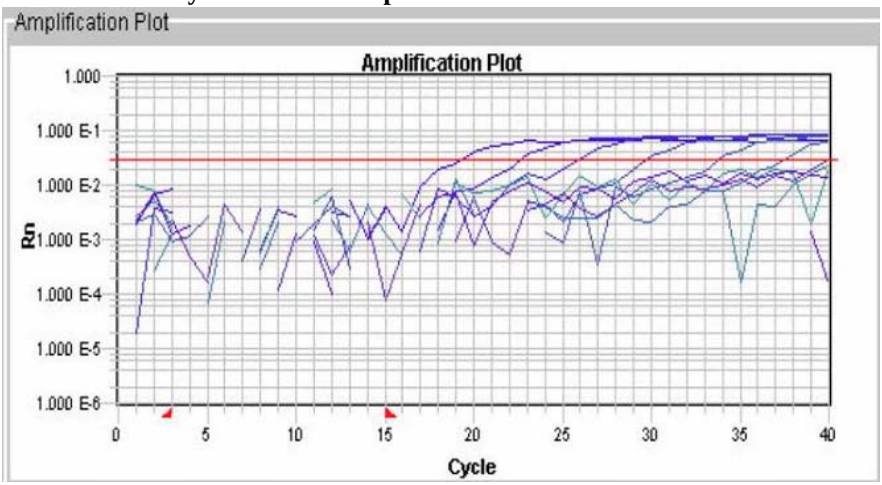


Figure 2. Analysis of Real-Time PCR Standard Curve for Enterovirus. The vertical axis is the Ct value; and the cross axis is the concentration (copies number).

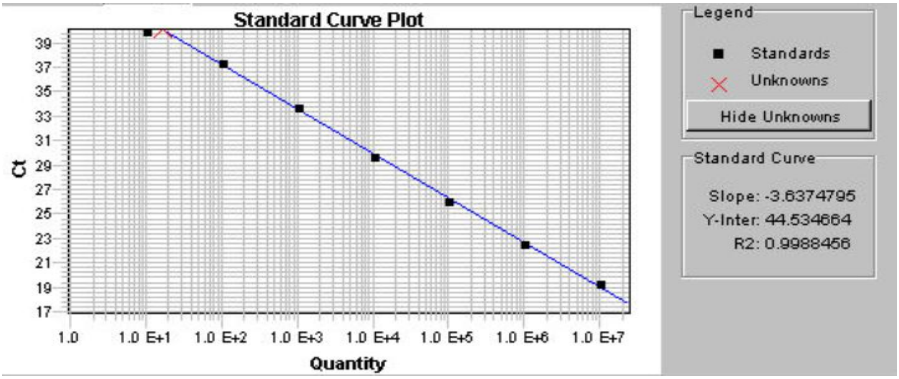
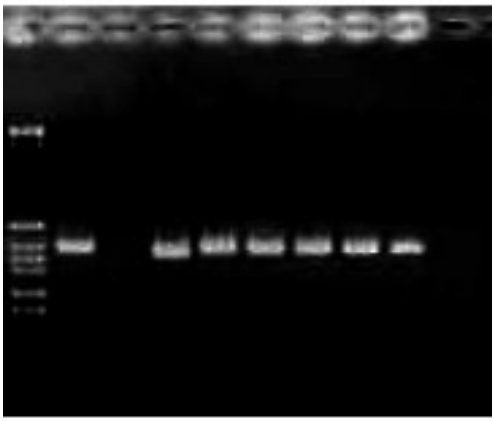


Figure 3. Comparing findings of analyses by RT-PCR and with findings of the qualitative analysis by real-time RT-PCR. Lane 1 is EV71; lanes 2, 3 and 4 are Eoho virus types 9, 4 and 6; lanes 5-8 are Coxsakievirus A2, A4, A6 and A16. M is marker 100 bp ladder; and N is negative controls.

M 1 2 3 4 5 6 7 8 N



RT-PCR: + - + + + + + + -

Real-time RT-PCR: + + + + + + + + --