行政院國家科學委員會專題研究計畫　成果報告

流感病毒預防及控制—圖譜、基因序列的建立(中南部地區)及相關研究

計畫類別：整合型計畫
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中　華　民　國 94 年 10 月 30 日

行政院國家科學委員會補助專題研究計畫 ■ 成 果 報 告
□期中進度報告

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成果報告類型(依經費核定清單規定繳交)：■精簡報告　□完整報告

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執行單位：中山醫學大學

中　華　民　國　九十四年十月 三十一 日

Abstract

Background: To establish the entire genome database of the influenza A virus, we require a simple method to amplify the full length gene segments of the virus. **Methods:** We designed primers based on the conserved regions of both the 5'- end and 3'-end of each gene segment in the influenza A virus. By optimizing the duration and temperature of denaturing, annealing, and extension, these primers can successfully amplify all full-length gene segments with RT-PCR. To test the accuracy, all amplicons were subjected for DNA sequencing with an autosequencer. **Results:** In total, we tested eighteen strains of influenza A virus which belonged to the H1N1 or H3N2 subtypes. We were able to amplify all eight segments of both subtypes in all tested strains. **Conclusions:** By using a new reverse-transcriptase with newly designated primers and PCR running conditions, we set up a novel protocol to amplify the entire genome of the influenza A virus. This method can used as a tool for amplification of all genes of H1N1 and H3N2 subtypes in the influenza A virus prior to analyzing their sequences, and to construct expression plasmids for further study.

Keywords: Influenza A virus; Entire genome; Thermoscript™ reverse transcriptase; RT-PCR

摘要

背景：為了建立一個 A 型流感病毒全基因庫，我們需要發展一套簡單的方法來增幅流感病毒各條全長基因。方法：我們利用 A 型流感病毒每段基因之 5'端及 3'端共同的保留區設計引子，經把核酸變性、煉合及延長等反應階段之時間及溫度設定最佳化後，可得到 A 型流感病毒各基因之全長產物。為了測試本方法之正確性。所有 PCR 產物均作基因序列分析。結果：我們共測試 18 株 H1N1 或 H3N2 亞型流感病毒，所有測試株之全長基因均可被增幅。結論：在本研究中，我們以新的 RT-PCR 方法，結合新的引子、新的條件來增幅 A 型流感病毒全部全長基因。本方法可用來增幅流感病毒全長基因外，更可在未來研究中幫助把基因構築到蛋白質表現載體。

關鍵字：A 型流感病毒、全長基因、Thermoscript™ 反轉錄酶、RT-PCR

1. Introduction

It was found in a recent study that the influenza A virus contains eight single-stranded RNA segments of a negative sense and encodes at least eleven proteins including PB1-F2 (Chen et al., 2001). The genome of the virus has been shown to undergo continuous variations through the influence of several mechanisms. These include not only rapid point mutation of the virus itself, but also genetic reassortment and gene recombining between different virus subtypes (Lin et al., 1994; Guo et al., 1995; Xu et al., 2004). These biological properties create further diversity of the influenza virus over time and thus cause difficulty in laboratory diagnosis, determination of virus strain selection for vaccine production, and prevention and control of the disease (Gavin and Thomson, 2004).

As with diagnostic methods for most viral diseases, many methods have been developed for identifying influenza viruses. In general, virus culture with Madin-Darby canine kidney cells and immunofluorescent staining is still the standard method applied in most clinical laboratories. Other methods like RT-PCR, real-time RT-PCR, nucleic acid sequence-based amplification, microsphere-based duplexed immunoassay, and DNA microarrays have also been established for molecular diagnosis and subtyping of influenza viruses in recent years (Claas et al., 1992; Lau et al., 2004; Stone et

al., 2004; Yan et al., 2004; Kessler et al., 2004; Phipps et al., 2004). Because of their particular diagnostic purposes, these methods only detect a small fragment of the genes for influenza virus. On the other hand, they cannot provide enough information to explain the genetic variations in the influenza virus. To study the gene variations of influenza virus, molecular diagnostic methods like ribonuclease protection assay, restriction fragment length polymorphism (RFLP), heteroduplex mobility assay (HMA), and single-strand conformation polymorphism (SSCP) have been well-established in recent years (Zou, 1997; Lopez-Galindez et al., 1988; Cox et al., 1989; Sakamoto et al., 1996; Ellis and Zambon, 2001; Saito et al., 2002; Lugovtsev et al., 2005). However, these methods cannot provide enough information to explain the gene reassortment and recombination of the virus. For this reason, we require an analysis of the entire gene of the influenza virus.

The vRNA segments of influenza A viruses have 13 and 12 nucleotide conserved sequences at both the 5′ and 3′ ends (Desselberger et al., 1980). The sequences are partially complementary to each other and form a panhandle structure (Bae et al., 2001). Many research groups used both sequences to design primer for synthesis of full length cDNA. By using these sequences to synthesize oligonucleotides, a full-length NS gene was cloned successfully (Baez et al., 1980). In addition, a new subtype of influenza virus was identified by using these sequences (Kawaoka et al., 1990). Practically, the conserved regions of both the 3′-end and 5′-end 11-14 nucleotides could not be used as universal primers for amplifying all eight segments of the entire gene of the influenza virus. Although modified oligonucleotides have been reported to amplify all genes of all subtypes of avian influenza viruses, the efficiency of their use has not been evaluated. Nor is it known whether they are also applicable to viruses over a period of years (Hoffmann et al., 2001).

In this study, we established a new protocol to amplify all of the genes of the influenza A virus in our laboratory. In addition, we also examined all the procedures from virus culture, RNA extraction, cDNA synthesis, and PCR, to optimize this protocol.

2. Material and methods

2.1. Virus

Human influenza virus strains (Table 1) were obtained from the Center for Clinical Virology and Research, Department of Clinical Laboratory, CSMU Hospital and from the Department of Pathology, Kaohsiung Medical Center, Chang Gung University and Memorial Hospital, Tainan. All the viruses were propagated for one or two generations in an MDCK cell line and stored at -70 ℃.

2.2. Primer design

Primers were modified from that described previously (Hoffmann et al., 2001) and were re-designed using GenBank sequence alignments (Table 2) (Altschul et al., 1990). To avoid the impurity of oligonucleotides, all primers were purified by PAGE.

2.3. Viral RNA extraction and cDNA synthesis

We used QIAamp Viral RNA Mini kit (Qiagen, Valencia, CA, USA) to extract vRNA from 150 μl of supernatants from infected MDCK cells. Reverse transcription was carried out using Thermoscript™ reverse transcriptase (RT) (Invitrogen, Carlsbad, CA, USA) and the primer Uni12 (M) 5’-AGCRAAAGCAGG-3’, which is complementary to the conserved 3′-end of all influenza A virus RNA segments. Briefly, 9 μl of the RNA preparation was mixed with 1 μl of 20 pmol of Uni12 (M), and 2 μl of 10 mM

dNTP mix to a volume of 12 μl. The mixture was incubated at 65 ℃ for 5 min. Then, the following constituents including 4 μl of 5X cDNA buffer, 1 μl of 0.1M DTT, 1 μl of RNaseOUT™ ribonuclease inhibitor (40 units/μl), 1 μl of sterile, distilled water, and 1 μl of Thermoscript™ RT (15 unit/μl) were added into the tube. The reaction was performed at 65 ℃ for 60 min. and was terminated by heating at 85 ℃ for 5 min.

2.4. PCR amplification

The conditions of PCR amplification were divided into three categories (Table 3). For HA, NP, NA, M, and NS, PCR Master Mix (GeneMark, Tainan, Taiwan) which contained 1.25 unit Taq DNA polymerase was used. For PB2, PB1, and PA, PCR Plus Master Mix (GeneMark, Tainan, Taiwan) which contained a mixture of recombinant Taq DNA polymerase and Pfu DNA polymerase was used. Briefly, the preparation of the reaction mixture contained 10 μl of PCR Master Mix or PCR Plus Master Mix, 10 μl of each of the primers, 2 μl (4 unit) of primer pairs and 1 μl of cDNA solution and added sterile, distilled water to reach a final volume of 50 μl. The amplification reaction was performed in a DNA thermal cycler (ABI 9700, Perkin-Elmer, Foster City, CA, US). The PCR conditions were summarized in Table 3. The amplified product was examined by 1.5% agarose gel electrophoresis and was stained with ethidium bromide.

2.5. DNA sequencing

To assess the accuracy of the amplicons, the nucleotide sequences of amplified genes from tested strains were determined. After separation of PCR products in agarose gels, bands containing the amplicons were excised and purified with a " Gel Extraction Kit" (Qiagen, Valencia, CA, US) for direct sequencing with cycle sequencing dye terminator chemistry (Perkin-Elmer, Foster City, CA). Sequences were analyzed with an ABI Prism 377A Autosequencer (Applied Biosystems). Comparison of nucleotide sequences was performed with the BLASTN program (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al., 1990).

3. Results

3.1. Selection of RT-PCR Primers

To synthesize cDNA from all genes of the influenza A virus, we concentrated on the 3'-termini of all segments of the virus and chose the first twelve nucleotide sequence to synthesize oligonucleotides. This region was highly conserved among all vRNA segments, except the fourth base which may be "A" or "G" in the cDNA of different influenza viruses. To make this primer more complementary, we substituted the fourth base of this primer from "A" to "R". To distinguish this primer from that described previously, we named this primer, Uni12(M).

3.2. Conditions of RT-PCR

For cDNA synthesis, we used Thermoscript™ RT rather than avian myeloblastosis virus (AMV) or murine leukemia virus (MMLV) RT. This enzyme can process its activity at 70℃ and is suitable for making long cDNA up to 10 kb from RNA with a complex secondary structure. For PCR, we optimized the conditions into three categories to amplify individual gene segments (Table 3). Examples of the amplicons are shown in Fig. 2.

3.3. Efficiency of gene amplification

To know whether our protocol was suitable for amplifying the influenza virus that was isolated from different years, in total, one hundred and forty-four genes from eighteen strains of the influenza A virus were isolated from 1996 to 2004. These were subjected to amplification of the full length genome of all segments. All genes could be amplified

successfully and appeared as specific bands in agarose gel electrophoresis, although some genes produced a weak band in the gel.

4. Discussion

Although sequencing is the most direct method of ascertaining the genetic information of the virus gene, it required a lot of labor and cost to derive the entire genome of a virus. In general, partial sequencing has usually been employed for molecular epidemiology studies of viral diseases (Suarez et al., 2003; Schrader and Suss, 2004). To generate a genomic database for the influenza virus, it is necessary to unravel the entire genome of the virus. In this study, we established a novel protocol that can be used to amplify the entire gene of the influenza A virus. We also examined each step, from the virus culture to the RT-PCR, to ascertain the factors that might affect gene amplification and how we would solve these problems.

To amplify all gene segments successfully, we had to passage the virus for at least two generations when the cytopathic effect had become clearly apparent (Data not shown). Especially in the case of the PB2, PB1 and PA genes, it was difficult to amplify all of the genes prior to sufficient virus propagation. This might have been due to the length of those genes being relatively long, thus making them easier to degrade during storage.

The former universal primer, Uni12, 5’-AGCAAAAGCAGG-3’ has been previously used to make cDNA from all genes of the influenza A virus (Gorman et al., 1990; Kawaoka et al., 1990; Hoffmann et al., 2001). However, sequences in GeneBank reveal that the fourth base of all genes of the influenza A virus can be G instead of A. To increase the efficiency of producing cDNA, we modified the cDNA primer into Uni12(M) 5’-AGCRAAAGCAGG-3’.

AMV RT and M-MLV RT are routinely used for cDNA synthesis, and their optimal reaction temperatures are 37 ℃ and 42 ℃, respectively (Fuchs et al., 1999). In fact, the efficiency of cDNA synthesis can be affected by thermal stablility, the affinities of RT for RNA templates and also the processivities of the enzyme. In a comparison between AMV RT and M-MLV RT, it was found that the resolving power of AMV RT in RNA-to-cDNA conversion was superior to that of M-MLV RT (Gerard et al., 2002). In addition, the efficiency was relatively low in M-MLV RT when target templates were rare in the RT reactions (Curry et al., 2002). In contrast, AMV RT binds much tighter to the template-primer during cDNA synthesis than M-MLV RT (Gerard et al., 2002). Practically, both M-MLV RT and AMV RT cannot effectively amplify the polymerase genes, PB2, PB1, and PA in the influenza virus. Since the length of PB2, PB1, and PA genes range from 2,233 to 2,241 bases, the virus RNA might possess a more complex secondary structure that was not complete denatured at 42℃. To solve this problem, we used a high temperature resistant RT, Thermoscript™ RT. Thermoscript™ RT is a recombinant avian reverse transcriptase that is lacking in essentially all RNase H activity. It has been engineered to have high thermal stability (up to 70°C) and is a good choice for RT-PCR in the case of exceptionally difficult templates. It can generate cDNA from 100 bp to >12 kb. This enzyme can copy RNA with a complex secondary structure at 65-70℃.

For viral gene that the length of genome exceeds 2 kb, e.g. PB2, PB1, and PA, the annealing of the oligonucleotide primers to the DNA templates becomes a critical step. Initially, we designed gene specific primers with lengths ranging from 18 to 20 bases to amplify the three polymerase genes. The

successful rates varied from gene to gene with a range from 50 to 70 % (Data not shown). This might have been due to the instability of the primers binding on the cDNA. To increase the efficiency and the stability of the binding force of the primers, we designed primers with lengths in the range of 25 to 27 bases and also increased the annealing temperature up to 58℃ (Table 3).

We tested eighteen strains of the influenza A virus, which represented up to nine years of isolates in this study. All amplicons could be easily detected as major bands in agarose gel electrophoresis. Although some PCR products might contain non-specific products, the major amplicon could easily be purified by gel extraction methods for further experiments. It implies that our method is applicable within nine-years of isolates despite the rapid mutation of influenza A virus over the years.

Mapping the whole genome sequence of influenza virus has many advantages. It can provide complete information for vaccine strain selection and development, antigenic drift and shift, interspecies transmission and evidence for drug resistance. We can also trace the origin of the virus accurately. For example, the relationship between the current strain and the 1918 Spanish flu virus (Reid et al., 2003; Reid et al., 2004).

In conclusion, the results of our work show that this method has high efficiency in amplification of the whole genome of H1 and H3 subtypes of the influenza A virus. Due to the identity of our designed primers relationship to the genomic sequences of different subtypes of avian influenza virus, our protocol might also be suitable for amplifying the whole genome of avian and other mammalian influenza viruses. Owing to the highly variability of genes in the influenza virus, PCR conditions need to be slightly modified between different strains of influenza viruses.

5. Self-accreditation

In this project, we established a novel method to amplify the whole genome of influenza A virus. The manuscript has been submitted to Journal of Virological Methods for reviewing. We totally sequenced twenty full-length genes which were deposited in NCBI GenBank database.

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Table 1. Strains tested in this study

Nomenclature	Abbreviation	Year of isolation	Subtype
A/Taiwan/117/96	TW117/96	1996	H1N1
A/Taiwan/118/96	TW118/96	1996	H1N1
A/Taiwan/252/98	TW252/98	1998	H1N1
A/Taiwan/929/99	TW929/99	1999	H1N1
A/Taiwan/930/99	TW930/99	1999	H1N1
A/Taiwan/2332/2001	TW2332/01	2001	H3N2
A/Taiwan/2984/2002	TW2984/02	2002	H1N1
A/Taiwan/2985/2002	TW2985/02	2002	H1N1
A/Taiwan/3640/2003	TW3640/03	2003	H3N2
A/Taiwan/4182/2004	TW4182/04	2004	H3N2
A/Taiwan/4183/2004	TW4183/04	2004	H3N2
A/Taiwan/30006/2001	TW30006/01	2001	H1N1
A/Taiwan/30017/2002	TW30017/02	2002	H1N1
A/Taiwan/30020/2002	TW30020/02	2002	H1N1
A/Taiwan/30022/2002	TW30022/02	2002	H1N1
A/Taiwan/30027/2002	TW30027/02	2002	H1N1
A/Taiwan/30005/2004	TW30005/04	2004	H3N2
A/Taiwan/31001/2004	TW31001/04	2004	H3N2

Table 2. Primers used for amplification of the entire gene

Primer	Sequence (5' to 3')*	Target segment	Size (bp)
Uni12(M)	AGCRAAAGCAGG	All	
PB2-1	AGCRAAAGCAGGTCAATTATATTCA	PB2	2341
PB2-2341R	AGTAGAAACAAGGTCGTTTTTAAACTA	PB2	
PB1-1	AGCRAAAGCAGGCAAACCATTTGAATG	PB1	2341
PB1-2341R	AGTAGAAACAAGGCATTTTTTCATGAA	PB1	
PA-1	AGCRAAAGCAGGTACTGATYCGAAATG	PA	2233
PA-2233R	AGTAGAAACAAGGTACTTTTTTGGACA	PA	
HA-1	AGCAAAAGCAGGGGAAAATA	HA	1778
HA-1778R	AGTAGAAACAAGGGTGTTTT	HA	
NP-1	AGCAAAAGCAGGGTAGATAA	NP	1565
NP-1565R	AGTAGAAACAAGGGTATTTTT	NP	
NA-1	AGCAAAAGCAGGAGTGAAAA	NA	1413
NA-1413R	AGTAGAAACAAGGAGTTTTTT	NA	
M-1	AGCAAAAGCAGGTAGATATT	M	1027
M-1027R	AGTAGAAACAAGGTAGTTTTT	M	
NS-1	AGCAAAAGCAGGGTGACAAA	NS	890
NS-890R	AGTAGAAACAAGGGTGTTTT	NS	

Codes for mixed bases position: R= A/G, Y= C/T.

Table 3. RT-PCR conditions for different gene amplifications

Gene	PCR conditions
cDNA synthesis	65 ℃ for 5 min; 65 ℃, 60 min; 85 ℃, 5 min
*PB2, PB1 & PA	94 ℃, 4 min; 94 ℃, 35 s; 58 ℃, 30 s; 72 ℃, 7 min for 30 cycles; 72 ℃, 7 min
HA, NP, M, & NA	94 ℃, 4 min; 94 ℃, 20 s; 43 ℃, 30 s; 72 ℃, 7 min for 30 cycles; 72 ℃, 7 min
NS	94 ℃, 4 min; 94 ℃, 20 s; 58 ℃, 30 s; 72 ℃, 7 min for 30 cycles; 72 ℃, 7 min

* *For PB2, PB1 & PA, PCR Plus Master Mix was used. For HA, NP, NA, M, and NS, PCR Master Mix was used.

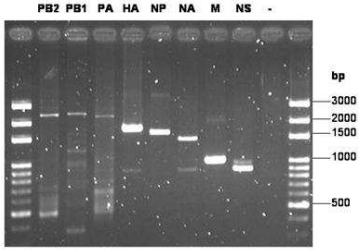

Fig. 1. Full length amplification of all eight segments by RT-PCR. RNA from A/Taiwan/118/96(H1N1) was isolated, and cDNA was synthesized with Uni12(M) primer. PCR reactions for each segment are performed by using the primer pairs shown in Table 2. Conditions for PCR are shown in Table 3. In "–" negative control, 1 ml sterile water instead of cDNA mixture was added to the PCR reaction. Five ml of 50 ml amplification products were electrophoresed on 1.5% agarose gel in Tris-boric/EDTA buffer and stained with ethidium bromide.

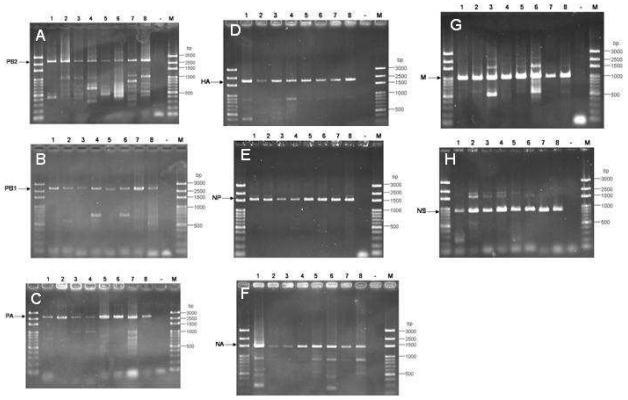

Fig. 2. Amplification of different segments in various conditions. Number 1-8 represents A/Taiwan/118/96, A/Taiwan/929/99, A/Taiwan/2332/2001, A/Taiwan/2984/2002, A/Taiwan/2985/2002, A/Taiwan/31001/2004, A/Taiwan/30005/2004, and A/Taiwan/30022/2002. "-" represents negative control. "M" represents molecular marker. The length of A. PB2= 2341 base pairs (bp), B. PB1= 2341 bp, C. PA= 2233 bp, D. HA= 1778 bp, E. NP= 1565 bp, F. NA= 1413 bp, G. M= 1027 bp, and H. NS=890 bp, which is indicated by the arrow in each panel.

Appendix: The nucleotide sequence data determined in this study will appear in DDBJ, EMBL, ISD and NCBI sequence databases under the accession numbers DQ249252, DQ249253, DQ249254,DQ249255, DQ249256, DQ249257, DQ249258, DQ249259, DQ249260, DQ249261, DQ249262, DQ249263, DQ249264, DQ249265, DQ249266, DQ249267, DQ249268, DQ249269, DQ249270, and