Comparison of Three Strategies for Sequencing Rabies Viral G Gene

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ABSTRACT

It is essential to rapidly, low-cost and precisely determining gene sequences of rabies virus. Reverse transcription-polymerase chain reaction can be used to identify rabies viral G gene sequences. In this study, we evaluated three methods, conventional RT-PCR and direct sequencing, adapter RT-PCR and sequencing with universal primers, conventional RT-PCR and cloning sequencing with universal primers, to detect rabies in animal brain homogenate. Four rabies isolates recovered from Fuyang city of Anhui province were diagnosed as positive using the fluorescentantibody test, rapid rabies enzyme immunodiagnosis methods and the mouse inoculation test. The results indicated that the adapter RT-PCR method and sequencing with universal primers is extremely well suited for sequencing rabies viral gene, which is rapid, cost-effective and precise compared with other two methods.

Abbreviations: RT-PCR: Reverse transcription-polymerase chain reaction, FAT: fluorescentantibody test, RREID: Rapid rabies enzyme immunodiagnosis methods, MIT: Mouse inoculation test. RABV: Rabies virus

Introduction

Rabies is one of the oldest and most feared zoonotic diseases, and has threatened to human health for more than 4000 years [1]. According to a WHO report, about 50,000 people die of this disease worldwide every year [2]. Rabies is acute and fatal viral encephalitis caused by a single stranded negative sense RNA virus belonging to the genus Lyssavirus of the family Rhabdoviridae. The RABV genome consists of a non-segmented, single-stranded negative-sense RNA of about

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12 kb, which encodes five viral proteins (3'-N-P-M-G-L-5') [3]. Twelve Lyssavirus species have been classified: Rabies virus (RABV), Lagos bat virus (LBV), Mokola virus (MOKV), Duvenhage virus (DUVV), European bat lyssavirus types -1 and -2(EBLV-1 and EBLV-2), Australian bat lyssavirus (ABLV), Aravan virus (ARAV), Khujand virus (KHUV), Irkut virus (IRKV), West Caucasian bat virus (WCBV)[4] based on the nucleotide sequences and deduced amino acid sequences similarities of the nucleoproteins (N), and Shimoni bat virus (SHIBV), Bokeloh bat lyssavirus (BBLV) and Ikoma lyssavirus (IKOV) awaiting classification. RABV [1, 5], classical rabies viruses, have a near world-wide distribution.

Laboratory techniques for diagnosis identify RABV with polyclonal antibodies or anti-nucleoprotein antibodies used in a direct FAT, which is recommended by both WHO and OIE, and is sensitive, specific and cheap [6], and RREID which detect virus antigen, virus isolation tests or MIT which detect live virus, RT-PCR diagnostic techniques and real-time PCR methods for the detection of rabies viral RNA have been developed and evaluated [7]. Despite the FAT gives reliable results on fresh specimens within a few hours in more than 95–99% of cases, and is sensitive, specific and cheap[6], this test can fail in decomposed samples or those with a small viral concentration[8, 9]. The classic MIT can lead to considerable delay, requires facilities for experimental animals, and is labor intensive. RT-PCR tests are rapid, sensitive and specific and when coupled to genetic sequencing can derive accurate genotyping and epidemiological information [10]. For a large number of samples, as in an epidemiological survey, RT-PCR can provide rapid results in specially equipped laboratories [6]. In human cases, RT-PCR allows for early detection of the infection and post-exposure prophylaxis for people exposed to the virus sources, genotyping information would have little direct consequence to patient treatment, but accurate genetic data could be used to establish epidemiological relationships and assist in the tracing of potential

contacts [10]. In animal cases, genotype--ing information is of greater importance as it may dictate the control method employed as a result of an outbreak [10].

In general, DNA sequencing is used to carry out the genetic typing of RABV, frequently used in molecular epidemiology. The main objective of this study was to develop one new method and evaluate for the detection of rabies viral RNA with RT-PCR and sequencing.

Materials and methods

1. Viruses

We used 4 RABV isolates (FY19-22), which isolated from Fuyang city of Anhui province [11]. Four brain samples were positive for rabies virus by FAT, RREID and MIT. The rabies virus antigen ELISA Kit and FITC-labelled monoclonal antibody were obtained from Gene Engineering Department of Wuhan Institute of Biological Products Co.,Ltd, China. The brains were frozen at -70°C until they were processed for virus isolation.

2.Viral RNA extraction and cDNA synthesis

Viral RNA was extracted from 0.1 g samples using 1ml TRIzol®(Invitrogen, USA), following the manufacturer's instructions. The extracted RNA was re-suspended in 20 μl of diethylpyrocarbonate treated water and stored at −70℃. Reverse transcription of viral RNA was performed in a volume of 100 μl using 1 μl extracted RNA, 4.5 μl DEPC-treated water, 10 units AMV Reverse Transcriptase (Promega, USA), 2 μl RT reaction buffer (25 mM Tris-HCl [pH of 8.3 at 42°C], 25 mM KCl, 5 mM MgCl2, 5 mM ditheothreitol, 0.25 mM spermidine), 100 μM of each dNTP, 10 units RNasin ribonuclease inhibitor (Promega) and 30 pmol random primers pd(N)6. RT reactions were incubated at 42℃ for 90 minutes, before heat-inactivation of the AMV Reverse Transcriptase at 95℃ for 5 minutes.

3. PCR and sequence determination of G gene

The complete G gene was amplified as two overlapping fragments, G1 and G2, using two pairs of primers in combination (Table 1 and Fig.1).

Fragment G1 was generated using primers M221/G781 or M13FM221/ M13RG781, corresponding to nucleotides (nts) 3000–4096 of the reference Pasteur virus (PV) strain. Fragment G2 was generated using primers MSL2/L2 or M13RMSL2 /M13FL2, corresponding to nts 3995–5753 of the PV strain (figure.1). The G1 and G2 were jointed to obtain complete G gene sequence (nts 3,310–4896 of the PV strain). We used three approaches to identify sequences. First, conventional RT-PCR and sequencing, we used specific primes M221/G781 and MSL2/L2 (figure.1a) for PCR reactions. Reactions of 50μl contained 1.5 μl cDNA, 5 μl 10X Ex Taq buffer, 20 pmol forward primer, 20 pmol reverse primer, 100 μM of each dNTP and 2.5 unit of ExTaq (TaKaRa). PCR conditions were as follows: an initial cycle of denaturation at 94°C for 4 minutes, annealing at 55°C for 50 seconds, extension at 72°C for 90 seconds, followed by 39 cycles of 94°C for 1 minute, 55°C for 50 seconds, 72°C for 90 seconds, and a final elongation at 72°C for 10 minutes. PCR products were visualized under UV light after electrophoresis

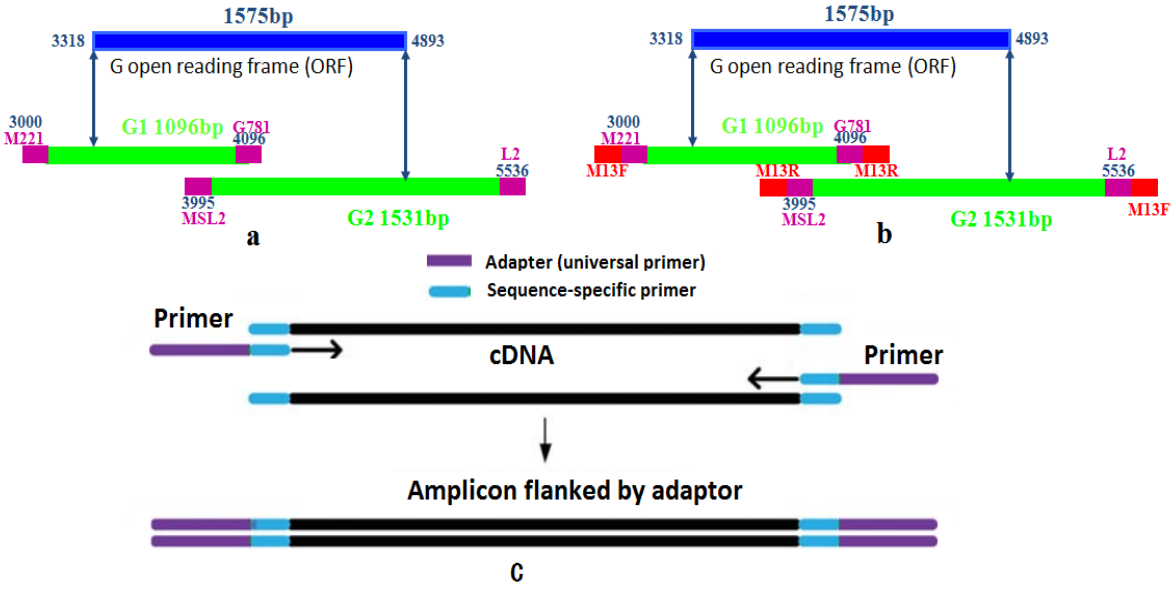

Figure.1. PCR strategies of G ORF for the rabies virus.

The principle of the adapter PCR is shown in Fig.1c.

through 1% agarose gels and visualized under UV light after staining with a nucleic acid stain (GoldView™, SBS Genetech, Beijing, China). The final PCR products were purified using Agarose Gel DNA Fragment Recovery Kit (TaKaRa) following the manufacturer's instruction. The purified products were sequenced using the Taq Big Dye Terminator Cycle Sequencing Ready Reaction Kit, according to the manufacturer's protocol, and the sequencing primers were M221/G781 and MSL2/L2. Reactions were analyzed on an Applied Biosystems 3730 DNA Analyzer.Second, adapter RT-PCR and sequencing (figure.1b), we used adapter primers M13FM221/M13RG781 and M13RMSL2/M13FL2 as same as the first PCR protocol and procedure. Amplicons were directly sequenced with universal primers M13F and M13R.

Table 1 Primers for PCR amplification and DNA sequencing

Method	PCR Primers	Sence	Viral locus	Nucleotide position [a]	Sequencing Primers
1	M221	+	M-G	3000-3019	M221
	G781	-	G	4077-4096	G781
	MSL2	+	G	3995-4016	MSL2
	L2	-	G-L	5730-5753	L2
2	M13FM221	+	M-G	3000-3019	M13F
	M13RG781	-	G	4077-4096	M13R
	M13RMSL2	+	G	3995-4016	M13R
	M13FL2	-	G-L	5730-5753	M13F

Note：

M13R: 5′-CAG GAA ACA GCT ATG ACC-3′,
M13F: 5′-TGT AAA ACG ACG GCC AGT-3′
M221: 5′-GGT GTA TCA ACA TGA ATT C-3′,
G781: 5′- CAT GTT CCG TCC ATA AG-3′
MSL2: 5′-TGG ATT TGT GGA TGA AAG AGG C-3′ ,
L2: 5′- CCG GCT CCT GTT TGA TTC AGA G-3′

[a] The positions of primers are based on the PV strain of rabies virus[12].

Third, cloning sequencing, the purified PCR products from the first method were

then ligated directly into the pMD18-T cloning vector system (TaKaRa) at 16 ℃ for 1 hour. The ligated product was transformed into competent E. coli DH5α cells by heat shock method, following the manufacturer's instructions. The transformed colonies were screened by both ampicillin as resistant marker and blue white color selection using X-gal, IPTG containing LB medium. Colonies were screened for inserts of the expected or similar size by PCR amplification using the universal primers M13F and M13R. Amplicons were sequenced using both M13F and M13R primers.

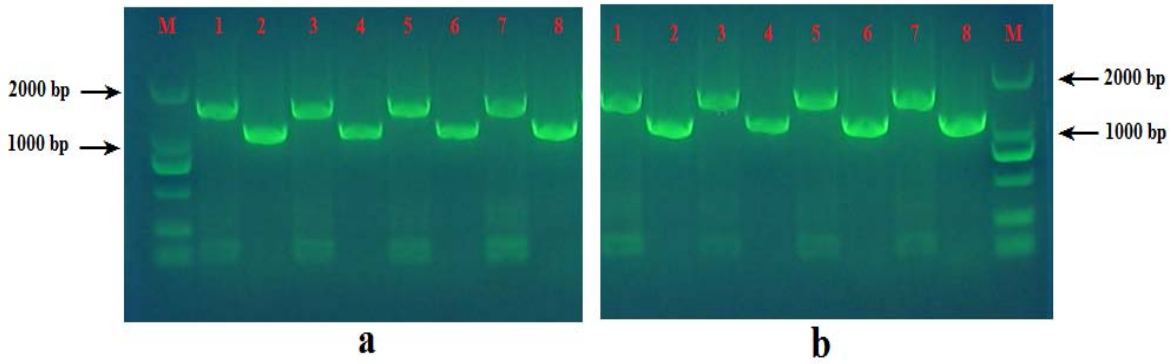

Figure.2. Result of RT-PCR using primer sets which amplified the G gene of rabies virus.
(1% agarose gels and visualized under UV light after staining with GoldView)
Lanes 1 and 2, 3 and 4, 5 and 6, 7 and 8 for samples represent FY19, FY20, FY21 and FY22, respectively.
In Fig.2a, lanes 1, 3, 5 and 7; 2, 4, 6 and 8 resulted from MSL2/L2 and M221/G781 primer sets, respectively.
In Fig.2b, lanes 1, 3, 5 and 7; 2, 4, 6 and 8 resulted from M13RMSL2/M13FL2 and M13FM221/M13RG781 primer sets, respectively. DL2000 DNA Ladder was used as the molecular weight marker (M).

Results

1 RNA extractions and RT-PCR

Amplification of 4 samples was obtained with 4 primer pairs for G gene yielding 1096 bp, 1531 bp; 1132 bp and 1567 bp products as expected, respectively (figure.2).

2 Purified amplified products and sequencing

Direct sequencing of a total of 1096 bases of fragments, G1, position 3000–4096, from 4 brain specimens was successfully obtained from RT-PCR, sample no. 2, 4, 6 and 8(figure.2a and figure.2b). PCR products from M221 and G781 primer sets and M13FM221 and

M13RG781 primer sets (product sizes of 1096 bp and 1132 bp, respectively) were used for sequencing with M221/G781(figure.3b and figure.3d) and M13F/M13R(figure.3a and figure.3c), respectively.

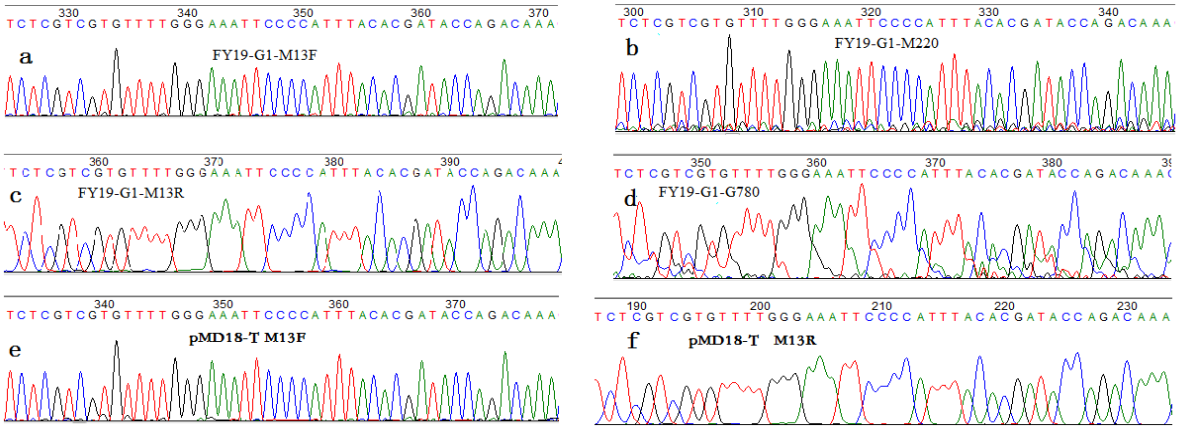

Figure.3. Partial Sequence (corresponding to nucleotides 3356-3402 of PV strain) maps of FY19 isolate. Fig.3b and Fig.3d show maps of sequencing with PCR primers.
Fig.3a and Fig.3c show maps of sequencing with universal primers.
Fig.3e and Fig.3f show maps of cloning sequencing.

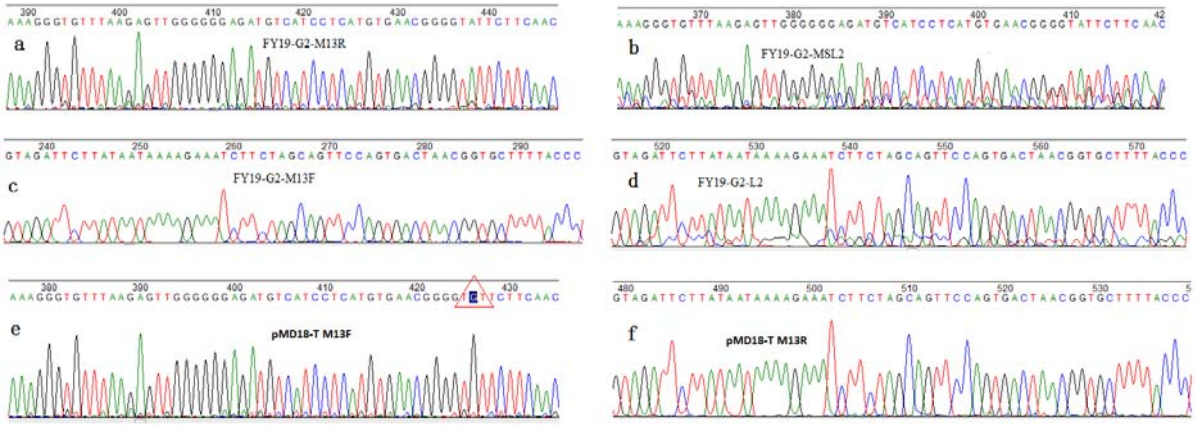

Figure.4. Partial Sequence maps of FY19 isolate
Fig.4b, c, f and Fig.4a, d, e corresponding to nucleotides 4396-4455 and 5054-5114 of PV strain, respectively.
Fig.4.b and Fig.4d show maps of sequencing with PCR primers.
Fig.4a and Fig.4c show maps of sequencing with universal primers.
Fig.4e and Fig.4f show maps of cloning sequencing.

Nucleotide sequencing of 1531 bases of fragments, G2, position 3995–5753, from 4 brain specimens was sets and successfully achieved from RT-PCR, sample no. 1, 3, 5 and 7(Fig.2). PCR products from MSL2 and L2 primer

M13RMSL2 and M13FL2 primer sets (product sizes of 1531 bp and 1567 bp, respectively) were used for sequencing with MSL2/L2 (Fig.4b and Fig.4d) and M13F/M13R(Fig.4a and Fig.4c), respectively.

G1 and G2 fragments were ligated into the pMD18-T cloning vector system, and then were sequenced using universal primers (M13F/M13R) in Fig.3e and Fig.3f, Fig.4e and Fig.4f, respectively. We showed only 46 bases, position 3356–3402 for FY19 isolate in Fig.3. In Fig.4a,4b and 4c; Fig.4b,4d and 4f, We showed only 59 bases(position 4396–4455) and 60 bases(position 5054–5114) for FY19 isolate.

Discussion

There are some distinct advantages and drawbacks to directly sequence a PCR product without first cloning the fragment. Direct sequencing is much quicker and doesn't show any PCR mutations. However, the 'mixed peaks' is often seen in which the bands start out fine, but later on become superimposed, when we directly sequence PCR products with PCR primers(Fig.3b and d, Fig.4b and d). Inefficient primers are sometimes good for PCR, but the same primers may fail in sequencing [13]. Because PCR is intrinsically an exponential process, and because it is usually carried well beyond completion, even rather poor primers will produce amplification in a PCR reaction [13]. However, sequencing is strictly linear, and is much more unforgiving of poor primers. Sequencing uses one primer, while PCR utilizes two. So we must purify PCR products to remove all residual PCR primers and unincorporated nucleotide. Direct PCR sequencing is rarely successful unless we can try to ensure that PCR primers satisfy the criteria of sequencing primers.

Sequencing with universal primer is an effective strategy for determination of cloned DNA fragments. We cloned PCR products into the pMD18-T cloning vector, and then sequenced plasmid by universal primers. We can get good quality of sequence in Fig.3e and Fig.3f, Fig.4e and Fig.4f. In fact, sequencing directly PCR products is better than sequencing a cloned PCR product, because when we

clone PCR products, we may have selected one with a base pair incorporation error, and thus when we sequence this plasmid. We find one mistake (Fig.4e) comparing with maps of sequencing directly PCR products because of the plasmid clones mutation. So we need clone those PCR products and sequence several plasmid clones. At any one nucleotide, most of the clones will be correct, and we can get the original sequence with no mutations. However, cloning PCR product and sequencing cost more money and time than directly sequencing PCR product.

The resolution of the gel normally decreases after perhaps 750-850 nucleotides during sequencing with PCR primers, which is normal for a Model 3730 sequencer. However, we are able to get good reads as much as 1000-1200 nt sequencing with universal primers. We find that sequencing maps with universal primers are better than sequencing maps with PCR primers from Fig.3 and Fig.4. These results show there are a direct relation between the resolution of sequence maps and the sequencing primers. Therefore, by combining the merits of direct PCR sequencing and cloning sequencing, we add an adapter (universal primer) to the 5' of sequence specific primer end (Fig.1b and c). Sequence-specific amplification of DNA depends on specific primer. The adapter (universal primer) is for sequencing. Because the presence of adapter sequences means that the molecules then can be selectively amplified by PCR, no bacterial cloning step is required to amplify the genomic fragment in a bacterial intermediate as is done in traditional sequencing approaches [14]. The method employs single molecule amplification prior to sequencing and therefore eliminates the need for prior cloning [15]. The structure of the adaptors evokes a PCR-suppression effect and provides a method for selective amplification of only those cDNA molecules that contain both adaptor sequence and sequence specific primer [16].

In conclusion, sequence data in combination with a suitable rabies genetic background, can be used to derive more precise epidemiological information and

assist in accurate tracing of the source of infection. The development of adapter RT-PCR and sequencing that has major advantages over direct PCR sequencing and cloning sequencing is described, as also is its ability to obtain a rapid and accurate diagnosis of RABV.

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