

EPIDEMIOLOGICAL SURVEY OF DIFFERENT RABIES VIRUS STRAINS IN IRAN

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Abstract

Rabies is widespread in all provinces of Iran. This study presents an investigation by a Polymerase Chain Reaction (PCR) technique for the purpose of rabies diagnosis and also Restriction Fragment Length Polymorphism (RFLP) for determination of the type of the rabies virus. In addition, PCR proves to be a powerful tool for molecular epidemiology studies allowing analysis of the infecting strains without prior cell culture adaptation. In this study, the type of rabies viruses isolated from 50 brain samples of various hosts from different provinces of the country was determined by restriction analysis (RFLP) on pseudogene ψ fragment as the most divergent genomic area. The results indicate that the viral strains belong to serotype/genotype 1.

Introduction

Rabies is a zoonotic viral disease which is caused by a group of neurotropic viruses of the family Rhabdoviridae, order Mononegavirales, genus *Lyssavirus* [19]. Rabies virus is a bullet-shaped virus with a diameter of 75-80 nm and a length of 100-300 nm, consisting of an unsegmented negative-sense single-stranded RNA molecule of about 12 Kb [5,6] which contains five genes N, P(M1), M(M2), G and L. These genes encode four proteins and a polymerase respectively. The nucleoprotein (N) produced by the N gene, is the most highly conserved among all rabies antigens, therefore it is an important fragment for rabies diagnosis [17].

The phosphoprotein and matrix protein, encoded

located within the viral genome between the N and G genes (Fig. 1) [17].

The P gene is overallly the least conserved in comparison to all coding regions, whilst the M gene is almost as conserved as N gene [6].

The glycoprotein is the only viral antigen that consistently induces virus neutralising antibodies. This protein is the most important antigen for immunization [17].

The remnant pseudogene ψ , separating the G and L cistrons, is one of the most divergent regions of the Rabies virus genome. It is a non protein encoding region which is highly susceptible to mutation and therefore illustrative for molecular epidemiological survey [18].

Rabies virus is capable of causing a lethal encephalitis in a wide variety of mammalian species. The principal animals currently involved are the stray dogs in Asia, Africa and Latin America, raccoon and

Keywords: Rabies; Molecular epidemiology; Typing; PCR respectively by the P(NS or M1) and M genes are

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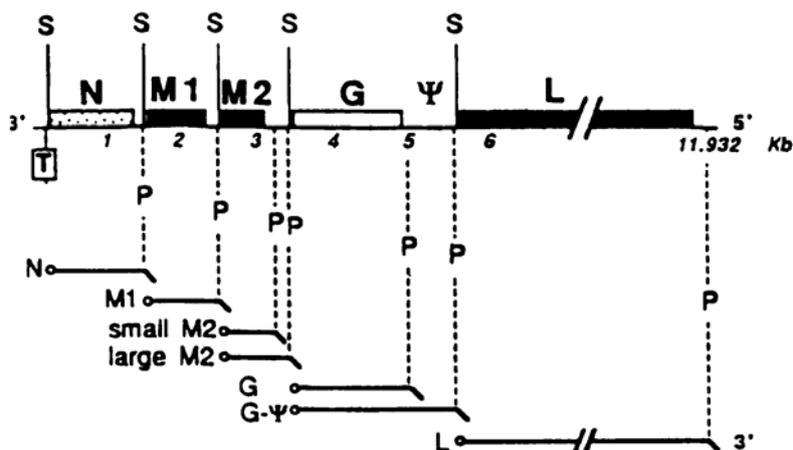


Figure 1. Rabies virus genome.

skunk in north America and red fox in Europe. Bat also constitutes an increasing global reservoir [13].

The numbers of individuals treated against rabies are 10,000,000 and over 50,000 human deaths are estimated to occur each year world wide [11,20]. Most of them are in developing countries (mainly in Asia).

On the basis of serologic and antigenic relationship, Rabies virus is classified into four serotypes. In addition, recently identified European bat Lyssaviruses (EBL1 and EBL2) have been classified in genotypes 5 and 6 [18]. Australian bat Lyssavirus (ABL) is the last strain which is described [3].

Rabies in Iran occurs in two forms, sylvatic and urban Rabies. The recorded data about rabies in Iran, show that all of the provinces of Iran are infected with rabies and the most incidences are in North, north-west, north-east, Esfahan and Fars provinces.

The highest rate of incidences reported in farm animals and dogs, therefore it has a significant role in economic loss in farm animals specially in north of Iran [15].

In this paper, we present an investigation by the PCR technique for the purpose of Rabies diagnosis and also RFLP for determination the type of the Rabies virus from the Rabies related viruses.

Materials and Methods

Among brain samples of various rabies suspected hosts received in 50% glycerol/H₂O (v/v) solution from different provinces of the country (Table 1), 50 rabies confirmed brain samples by FAT were collected and conducted to subsequent molecular studies.

RNA Extraction

Total brain tissue RNA was extracted by Trizol reagent (Gibco BRL, Life Technologies Inc.) according to the manufacturer's instruction and stored at -70°C.

Oligonucleotide Primers

Primers used in this study were synthesized according to previous studies of Sacramento *et al.* [12] located within glycoprotein and polymerase genes of the Rabies virus, spanning the pseudogene. These oligonucleotide primers were named G and L primers consisting of the following sequences:

G : 5'-GACTTGGGTCTCCCGAACTGGGG-3'

L : 5'-CAAAGGAGAGTTGAGATTGTAGTC-3'

G primer serves as forward and L as reverse for PCR amplification.

Another set of primers was also used for diagnostic purposes:

N1: 5'-TTTGAGACTGCTCCTTTTG-3'

N2: 5'-CCCATATAGCATCCTAC-3'

This primer set is located in the most conserved region of the rabies genome [12].

cDNA Synthesis

1 µl of total brain RNA (1 µg) was hybridized in 5 µl total volume to G primer with 5 µM final concentration. The mixture was heated in a water bath at 90°C for 3 min with immediate cooling on ice, then 5 µl reaction buffer was added to it with final concentration of RNAsin (MBI, fermentas) 1 unit/µl, 10 unit/µl MMulv-RT (Gibco BRL, Life Technologies Inc.), 1 mM dNTPmix, 10 mM DTT, 50 mM Tris-HCl pH=8.3, 75 mM KCl and 3 mM MgCl₂. The reaction mixture was incubated at 37°C for 1 h.

Table 1. 50 Rabies brain samples of various hosts from different provinces of Iran

Province	Animal							
	Dog	Camel	Cow	Beef	Sheep	Donkey	Wolf	Jackal
Ardebil	1							
Sistan & Balouchestan		4	4					
Esfahan	2		1				1	
Tchaharmohal va Bakhtiari	2		1	1				
Gilan	1		1	1				
Bushehr						1		
Kerman				1	1			
Kohkiloye & Boyerahmad				1				
Kermanshah	1		1				1	
Kordestan	1							
Fars	1		5	1			2	
East Azarbaijan	4							1
Hamedan	1							
Khorasan	1		2		1			2
Lorestan	1							

PCR Reaction

cDNA containing solution was brought to 50 μ l with TE buffer and 5 μ l of this solution was used for PCR reaction with following conditions:

dNTP mix 1 mM, MgCl₂ 1.7 mM, Tris 50 mM, KCl 50 mM, G-L primer set with final OD 0.05, one unit Taq polymerase (Gibco BRL, Life Technologies Inc.) to 50 μ l final volume. The PCR program started with 5 cycles of 94°C for 60 sec, 49°C for 90 sec, 72°C for 90 sec followed by 30 cycles of denaturation in 94°C for 30 sec, annealing at 49°C for one min, extension at 72°C for 90 sec with a 3 min pre-heating at 94°C and 10 min final extension at 72°C, in a thermal cycler (Master Cycler, eppendorf No. 5332 00605).

Polymerase chain reaction for diagnosis of rabies in the samples was treated in the same time and thermal conditions with an exception for annealing temperature which was 45°C.

Restriction Analysis (RFLP)

10 μ l aliquots of PCR product was subjected to restriction enzyme analysis in a 20 μ l reaction mixture containing a units of *Hind*III restriction endonuclease (Cinnagen Inc. Tehran), Tris-HCl 50 mM pH=8.0, MgCl₂ 10 mM, NaCl 50 mM, incubated for 1 h at 37°C in a water bath. The results were analyzed in a 2% agarose gel electrophoresis in a 0.5x TBE buffer based on Sambrook *et al.* [14].

Results

Total RNA extracted from Rabies virus infected brain samples. Preparation of cDNA performed preliminary by G priming. The products subsequently amplified by PCR using both members of the G-L primer set; Figure 2a shows PCR results containing an approximately 900 bp fragment present in all products, comparing with a PV strain as positive control and a 100 bp ladder (Gibco BRL, Life Technologies Inc.).

Further endonuclease restriction studies on the products confirmed the specificity of the amplified fragments. Also refused the probable presence of nonspecific co-migrating PCR products.

Previous analysis of several Rabies and Mokola virus sequences by Sacramento [12] and Tordo [17] allowed classification of the strains into homologous groups. These groups may even separate members of the same genotypic group; Pasteur Virus (PV-11)/Louis Pasteur Virus (PAS) and Evelyn-Rokitnicki-Abeleseth (ERA) in group I and Challenge Virus Strain (CVS)-11, Avirulent Orsay 1 (AVO-1) and Pitman-Moore (PM) in group II. Using a panel of 4 restriction enzymes *Bam*H1, *Hind*III, *Hinc*II and *Pst*I, it is easy to distinguish the five groups by simple observation of the cleavage of the G-L amplified fragment (within pseudogene region) in ethidium bromide agarose gel [12].

Figure 2b shows the cleavage pattern of the G-L amplified segment with the endonuclease. The first

endonuclease of choice was *Hind*III by which all study samples were cut giving a pattern consisting of 700 and 200 bp bands. These results indicate that the studied viral strains so far are belong to PV, PAS group which in turn are located in the first genotypic group of the Rabies virus.

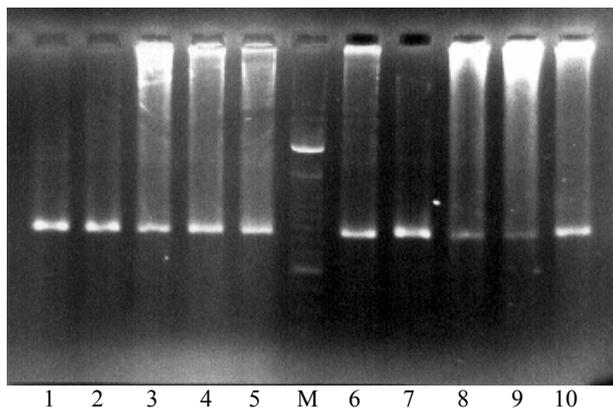


Figure 2a. Results from viral cDNAs undergone PCR. Products contain a single band about 900 bp according to size marker, obtained from G-L primer set. Lanes 1-10 PCR product of G-primed cDNAs. Lanes 1, 4, 5, 6 are samples isolated from dogs of Ouroumieh, Farsan, Esfahan and Marvdasht; Lanes 2 and 3 are samples isolated from camels of Iranshahr; Lanes 7, 8, 9 and 10 are samples isolated from Jieroft, Iranshahr and Masal respectively. Lane M is 100 bp ladder.

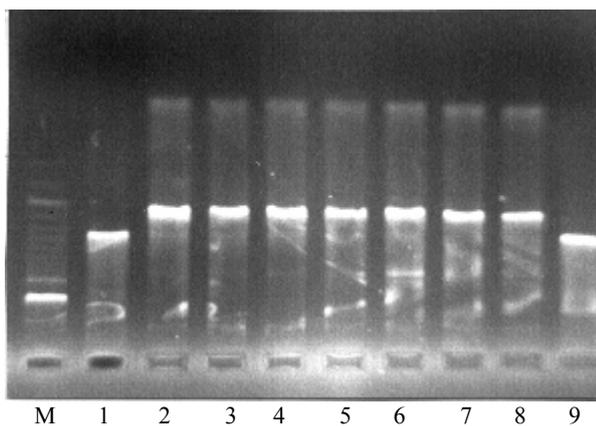


Figure 2b. Restriction enzyme digestion of PCR products. *Hind*III digestion of PCR products, using G-L primer set cleaves products of members belonging to serotype/genotype1 in to two bands with 700 and 200 base pair length. Lanes 2-8 digested PCR product of G-L primer set, Lanes 1 and 9 undigested PCR product of G-L primer set, Lane M is 100 bp ladder.

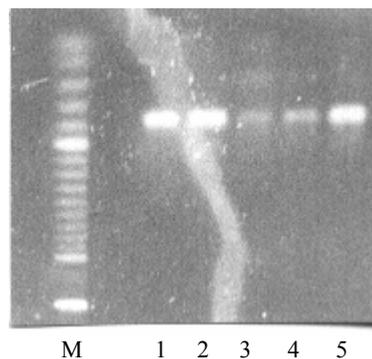


Figure 3. PCR product obtained from N1-N2 primer set. Bands are moving between 400 and 500 bp of size marker. Different intensities are due to severity of infection in the samples. Lanes 1-5: N1-N2 PCR products. Lane M is 100 bp size marker.

Products obtained from PCR on Rabies cDNAs by N1-N2 set on a 1.5% agarose gel oriented a band moving between 400 and 500 bp bands of the size marker, indicating presence of the virus in the samples, Figure 3.

Discussion

Official reports of human rabies and animal rabies through the country show that this disease is widespread in all provinces in Iran specially in the north, north-west and north-east regions of the country and has a significant role in economic loss and social disruption. During the year 2000 all of the provinces of Iran, were infected by rabies (urban and sylvatic rabies). A total of 74168 exposed persons received post-exposure treatment and 9 human rabies cases occurred in Iran. A number of 398 specimens were found positive for rabies [2]. These data show that rabies is a serious problem in Iran and an epidemiological survey about the different strains of rabies virus, can help for prevention and control of the disease. The pattern obtained from restriction endonuclease analysis of PCR products produced by G-L primer set shows that all 50 samples belong to genotype 1. Collection of these samples from various provinces of the country in turn shows that results of the current study are in accompany with results of monoclonal antibody studies in our center (WHO Collaborating Center for Reference and Research on Rabies), indicating widespread presence of the disease in the country.

Previous studies on rabies pseudogene have shown that this non-protein coding region of the genome located in the G-L interregion sequence is carrying the most divergent sequence between rabies genes. Furthermore, this highly variable genomic area which is

greatly susceptible to mutation and subsequently more likely to represent natural evolution of the virus, is carrying restriction sites corresponding to rabies and rabies-related variants, including genotype 1. In this respect, our current work shows that all samples carrying *HindIII* restriction site at a similar location are from genotype 1, also shows direct correlation among serotypic studies of this center with MAbs on rabies during 1990s, which have shown the presence of serotype 1 (genotype 1) in the country.

Vaccines currently being administered for pre and post-exposure treatment of rabies in the country are totally raised against members of serotype (genotype) 1. This issue explains the necessity of epidemiological surveys of the same category regularly in the country, since the appearance of non-PV strains (variants other than what are in existence now) could potentially result in failure of treatment. However, as the molecular biology techniques in comparison with serologic technique are more rapid and accurate [10], they can be placed in prior preference in such surveillances.

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