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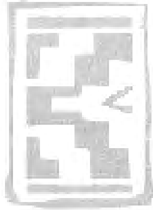

A nucleotide-specific polymerase chain reaction assay to differentiate rabies virus biotypes in South Africa

L.H. NEL[1]*, J. BINGHAM[2], J.A. JACOBS[1] and J.B. JAFTHA[1]

ABSTRACT

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Antigenic and nucleotide sequence analyses have shown that two distinct biotypes of rabies virus are circulating in South Africa. One of these typically infects members of the family *Canidae*, while the other comprises a heterogeneous group of apparently indigenous viruses, infecting members of the *Viverridae* family. In recent times, it has become evident that a considerable amount of cross-infection may occur and the manifestation of viverrid rabies in non-viverrid animals in particular appears to have become more commonplace. Consequently, the need to rapidly distinguish between rabies virus biotypes has become increasingly important in efforts to monitor the epidemiology of rabies in the southern African region. In this study, a nested polymerase chain reaction (PCR) assay was developed to distinguish between these two groups of rabies viruses. Consensus oligonucleotides were used to amplify the cytoplasmic domain of the rabies virus glycoprotein and the adjacent intergenic region. The resultant amplicon was subsequently used as template in a second round hemi-nested PCR in the presence of type-specific primers, thereby successfully generating amplicons of characteristic size for each biotype.

Keywords: Rabies, virus biotypes

INTRODUCTION

Two distinct biotypes of rabies virus are endemic in South Africa. One of these biotypes is indigenous to southern Africa and is mainly associated with different species of the family *Viverridae*, such as mongoose species (*Cynictis* spp.) and genets (*Genetta* spp.) (Nel, Thomson & Von Teichman 1993). Phylogenetic analysis identified different clusters of these "viverrid" viruses, which were found to correlate with distinct geographic origins (unpublished results). The other biotype, referred to as the "canid" biotype, is closely related to the European rabies virus strains, and is maintained by members of the family *Canidae*, viz. bat eared foxes (*Otocyon megalotis*), jackal species (*Canis* spp.) and domestic dogs (*Canis familiaris*) (Von Teichman, Thomson, Meredith & Nel 1995).

Currently, laboratory confirmation of rabies in South Africa is based on the fluorescent antibody test (FAT). Rabies biotypes are distinguished using a panel of anti-nucleocapsid-specific monoclonal antibodies (King, Meredith & Thompson 1993). Although sequence analysis has been employed to determine the epidemiological relationships among different rabies viruses (Sacramento, Badrane, Bourhy & Tordo 1992; Smith, Orciari, Yager, Seidel & Warner 1992; Nadin-Davis, Casey & Wandeler 1993; Smith, Yager & Orciari 1993; Von Teichman *et al.* 1995), its application to routine diagnosis may prove very costly. In developing new generation diagnostic techniques, amplification of viral nucleic acid sequences by the polymerase chain reaction (PCR) (Mullis & Faloona 1987)

[1] Department of Microbiology and Plant Pathology, Biological and Agricultural Sciences, University of Pretoria, Pretoria, 0002 South Africa

[2] Onderstepoort Veterinary Institute, Private Bag X5, Onderstepoort, 0110 South Africa

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enables rapid and reliable diagnosis and strain differentiation for rabies diagnosis (Ermine, Larzul, Ceccaldi, Guesdon & Tsiang 1990; Marschall, Schuler, Boswald, Helten, Hechtfischer, Lapatschek, & Meier-Ewert 1995; Sullivan & Akkina 1995; Vangrysperre & Clercq 1996). Recently, Nadin-Davis, Huang & Wandeler (1996) demonstrated the use of strain-specific oligonucleotides in a PCR method to discriminate between the racoon rabies virus and the indigenous strains in Ontario. Thus, in contrast to genome sequencing, type-specific PCR assay presents a rapid method for characterizing virus isolates. Furthermore, PCR-based diagnosis is more sensitive than the conventional immunological tests: Kamolvarin, Tirawatnpong, Rattanasiwamode, Tirawatnpong, Panpanich & Hemachudha (1993) were able to detect viral RNA (as little as 8 pg) in brain samples left at room temperature for an extended period of time.

Viruses typically associated with viverrid hosts make up a very heterogenous and seemingly indigenous group, composed of several different genetic clusters of virus, in contrast to the less variable canid viruses which are more closely related to viruses from Europe (Von Teichman *et al.* 1995). In order to correctly understand rabies epidemiology in South Africa, it is necessary to distinguish between the different biotypes of the virus. Although the biotypes are associated with different host ranges, overlapping of hosts does occur with an apparent increase in frequency (Nel, Jacobs, Jaftha & Meredith 1997). Thus, the aim of this investigation was to develop a PCR assay whereby canid and viverrid viruses can be distinguished rapidly, irrespective of host species or geographic origin. To this end, discriminating oligonucleotides were designed which, when used in a competitive hemi-nested reaction, gave amplicons of distinctly different sizes enabling rapid differentiation of the virus biotypes.

MATERIALS AND METHODS

Rabies virus isolates

Ten viverrid virus isolates were selected to be representative of all the known genetic subtypes, specific to different geographical regions of South Africa (unpublished results). Viruses of the canid biotype are closely related (Nel *et al.* 1993) and six isolates were selected to include the most diverse among these (Table 1). Sequence comparison of the above-mentioned isolates was used in order to design biotype-specific primers. In addition, a collection of 42 rabies-positive isolates were obtained from the Rabies Unit of the Onderstepoort Veterinary Institute (Table 1). These isolates were previously characterized by a panel of nucleoprotein-specific monoclonal antibodies and were used to assay the efficacy of the hemi-nested PCR.

Design of the intergenic biotype-specific primers

The biotype-specific primers were based on sequences of the cytoplasmic domain of the rabies virus glycoprotein and the G-L intergenic region (Sacramento *et al.* 1991; von Teichman *et al.* 1995; Nel *et al.* 1997). For optimal sequence alignment, sequences of the cytoplasmic domain and of the G-L intergenic region were independently aligned using the automatic sequence alignment function of the DAPSA computer package (Harley 1992). From these alignments, a consensus sequence was determined for the viverrid and canid biotype viruses. These consensus sequences were subsequently aligned in order to identify areas of sequence disparity and type-specific primers were designed to anneal specifically to their respective genotypes. The canid-specific and the viverrid-specific primers were designated $P_{can}(+)$ and $P_{viv}(+)$ respectively and were chemically synthesized (Boehringer, Mannheim) at a scale of 0,2 μM.

Rabies virus typing by a competitive hemi-nested PCR using type-specific primers

Viral RNA extraction, cDNA synthesis, PCR amplification and purification of PCR amplicons were carried out according to previously described methods (Sacramento *et al.* 1991; Von Teichman *et al.* 1995). The virus typing was performed in a single reaction tube in a second round competitive hemi-nested PCR. The template for this reaction was generated by a first round of amplification using the G-L primer pair (Sacramento *et al.* 1991) which amplifies a 850 bp product encompassing the highly variable cytoplasmic domain of the glycoprotein and the G-L intergenic region (position 4665–5520, of the Pasteur virus sequence [Tordo, Poch, Ermine, Keith & Rougeon 1986]). The hemi-nested PCR was carried out in a 50 μℓ reaction mixture containing 5 μℓ of the first round product (diluted 1:500 in sterile distilled water), 100 mM of each dNTP, 1,5 mM $MgCl_2$, 50 mM KCl; 10 mM Tris-HCl pH 9,0; 0,1 % Triton X-100, 100 pMol of the L(-) primer and 50 pMol of both $P_{can}(+)$ and $P_{viv}(+)$ in the presence of 0,25 U of Taq DNA polymerase. Following initial denaturation of 5 min at 95 °C, the samples were subjected to 30 cycles of denaturing at 94 °C for 45 s, annealing at 60 °C for 30 s and elongation at 72 °C for 1 min. Amplicons were visualized on 1,5 % agarose gels (Sambrook, Fritsch & Maniatis 1989).

RESULTS

Design of biotype specific primers

In order to design biotype-specific primers, a consensus sequence for both the genotypes were determined (Fig. 1). In positions where a specific nucleotide

TABLE 1 Rabies virus isolates, host species of isolation, Mab reactivity and biotype-specific PCR result

Virus isolates	Host species	Mab typing	Size of amplified product	
			650 bp	400 bp
701/92[a]	*Canis mesomelas*	V	✓	
636/90[a]	*Galerella pulverulenta*	V	✓	
926/90[a]	*Suricata suricatta*	V	✓	
421/92[a]	*Canis familiaris*	V	✓	
5/91[a]	*Canis mesomelas*	V	✓	
668/92[a]	*Atilax paludinosus*	V	✓	
158/91[a]	*Canis mesomelas*	V	✓	
298/90[a]	*Felis lybica*	V	✓	
256/90[a]	*Otocyon megalotis*	V	✓	
669/90[a]	*Cynictis penicillata*	V	✓	
612/94	*Canis mesomelas*	V	✓	
461/94	*Felis lybica*[b]	V	✓	
558/95	*Suricata suricatta*	V	✓	
522/95	*Felis serval*	V	✓	
567/94	*Atilax paludinosus*	V	✓	
610/94	*Felis lybica*	V	✓	
707/92	*Genetta*	V	✓	
782/94	*Bovine*	V	✓	
708/94	*Suricata suricatta*	V	✓	
1088/94	*Feline*[b]	V	✓	
500/94	*Suricata suricatta*	V	✓	
928/94	*Galerella sanguinea*	V	✓	
35/94	*Atilax paludinosus*	V	✓	
866/94	*Galerella sanguinea*	V	✓	
427/94	*Felis lybica*	V	✓	
532/95	*Canis familiaris*	V	✓	
639/93	*Feline*[b]	V	✓	
919/95	*Canis familiaris*	V	✓	
689/94	*Suricata suricatta*	V	✓	
1716/80	*Feline*[b]	V	✓	
262/95	*Suricata suricatta*	V	✓	
E17	*Cynictis penicillata*	V	✓	
E27	*Cynictis penicillata*	V	✓	
484/94	*Canis familiaris*	V	✓	
637/90[a]	*Xerus inauris*	C		✓
spu152[a]	*Atilax paludinosus*	C		✓
HR1/79[a]	*Genetta*[b]	C		✓
31/96[a]	*Canis familiaris*	C		✓
Sk0006[a]	*Cynictis penicillata*	C		✓
NBA2[a]	*Otocyon megalotis*	C		✓
77/93	*Felis lybica*	C		✓
1265/80	*Canis familiaris*	C		✓
774/95	*Otocyon megalotis*	C		✓
45/94	*Canis mesomelas*	C		✓
718/94	*Bovine*	C		✓
583/94	*Otocyon megalotis*	C		✓
460/94	*Otocyon megalotis*	C		✓
NBA5	*Canis mesomelas*	C		✓
19518/91	*Galerella sanguinea*	C		✓
22574/92	*Civettictis civetta*	C		✓
902/95	*Panthera leo*	C		✓
906/80	*Feline*[b]	C		✓
E64	*Cynictis penicillata*	C		✓
E107	*Cynictis penicillata*	C		✓
522/94	*Otocyon megalotis*	C		✓
716/95	*Suricata suricatta*	C		✓
487/94	*Otocyon megalotis*	C		✓

[a] Sequence information is available, used to design primers
[b] Exact species not positively identified

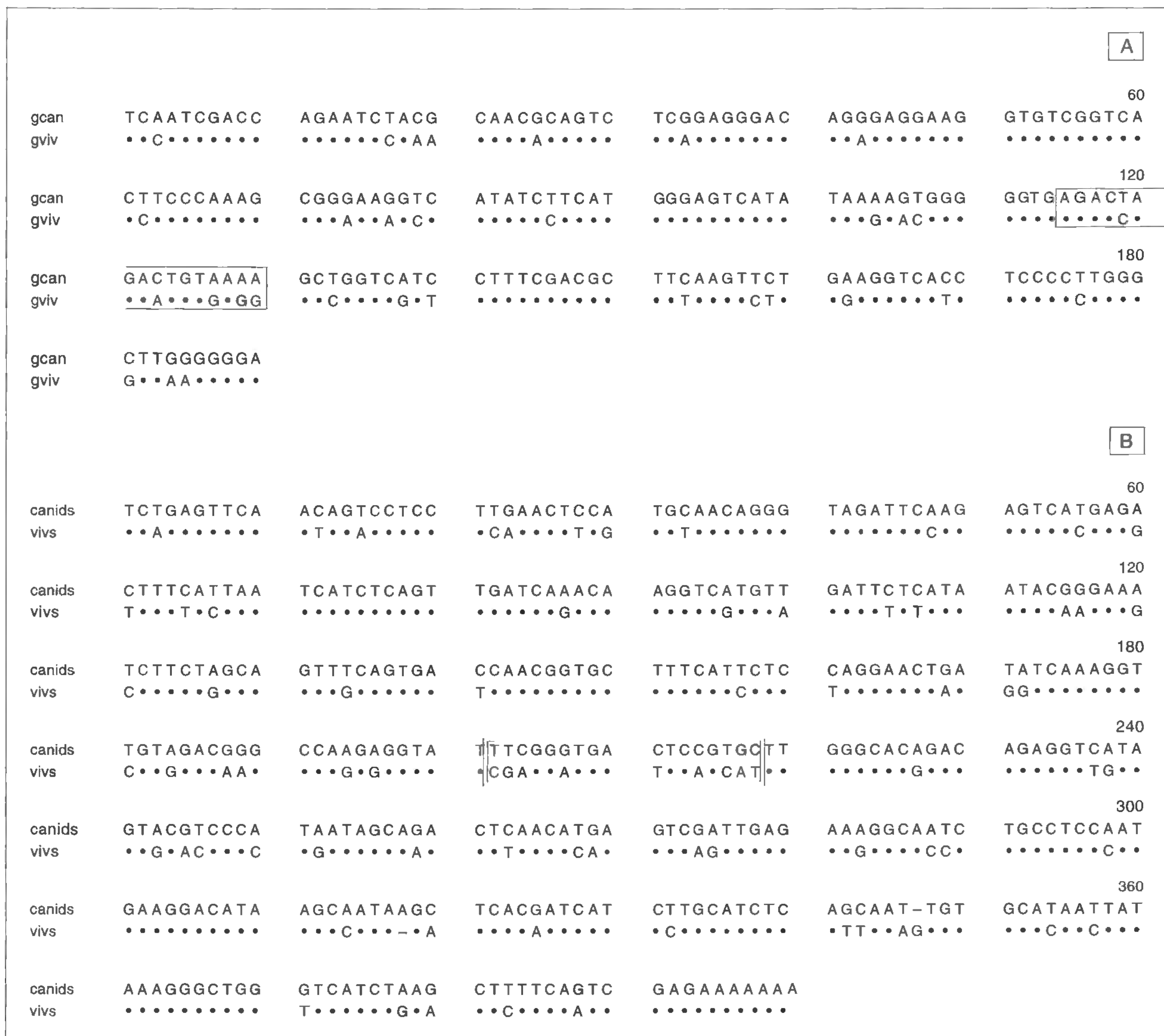

FIG. 1 Alignment of the consensus cDNA sequences of (A) the cytoplasmic domain and (B) the G-L intergenic region of the canid and viverrid biotypes of rabies viruses. Areas of sequence variation are indicated with inclusion of the appropriate nucleotide whereas identical nucleotides are indicated by dots (•). Priming sites are shown for the viverrid-specific primer (single box) and the canid-specific primer (double box)

occurred in more than 90% of sequences investigated, it was considered to be the consensus nucleotide. A comparison between the two biotype-specific consensus sequences revealed a 17% nucleotide difference when considering the cytoplasmic domain. A similar comparison of the consensus sequences of the intergenic region revealed a 20% nucleotide difference. Areas of sequence variation within the consensus sequence were subsequently considered for potential priming sites (Fig. 1). The first potential primer position was located within the cytoplasmic domain, approximately 204 bp downstream from the priming site of the G(+) primer. Within this region five nucleotide differences were present, three of which were present near the 3' end of the site. This site was selected as a priming site for a viverrid-specific primer and the primer was designated P_{viv} (+) (Table 1). A wobble base (A/G) was included at position two within this primer, considering that 12% of isolates analysed displayed an adenine (A) at this position, compared to a guanine (G) residue in the remainder of the isolates. The comparison of the intergenic consensus sequences provided the second potential priming site for a canid-specific primer. This region contained 9 nucleotide differences in close proximity (Fig. 1). The primer sequence P_{can} (+) is shown in Table 1. When aligned on the viverrid consensus sequence template, the final eight nucleotides at the 3' end differs in five positions, suggesting that this primer should be highly suitable for discrimination

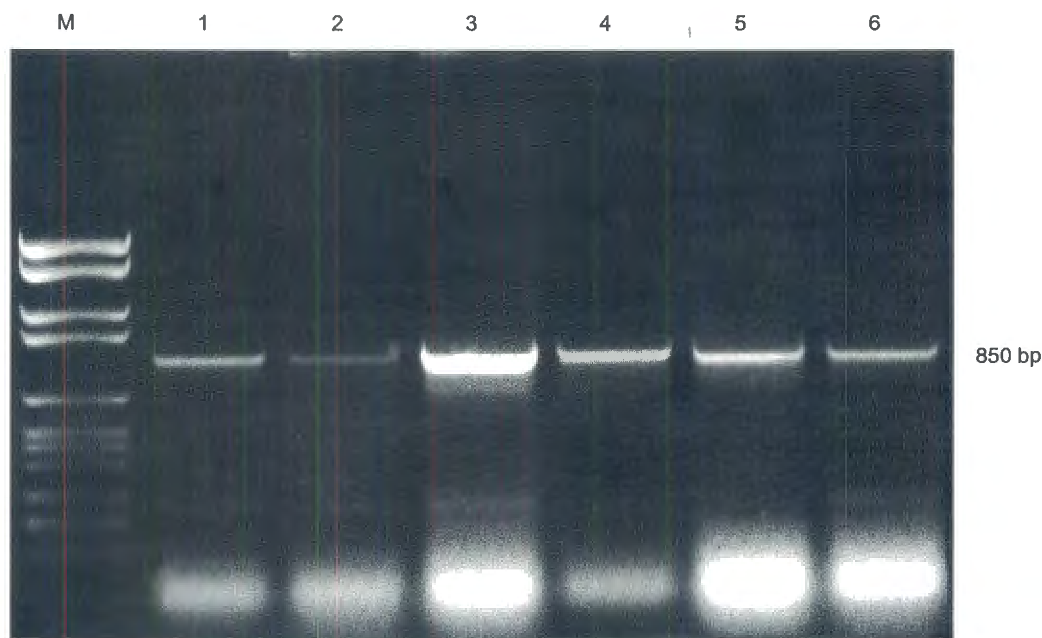

FIG. 2a Examples of first round amplicons using the G-L primer pair and virus-specific cDNA templates, prepared as described in Materials and Methods. DNA molecular weight marker VI (Boehringer Mannheim) is shown in lane M, while the amplicons specific to typical viverrid and canid viruses are shown in lanes 1–3 and 4–6, respectively

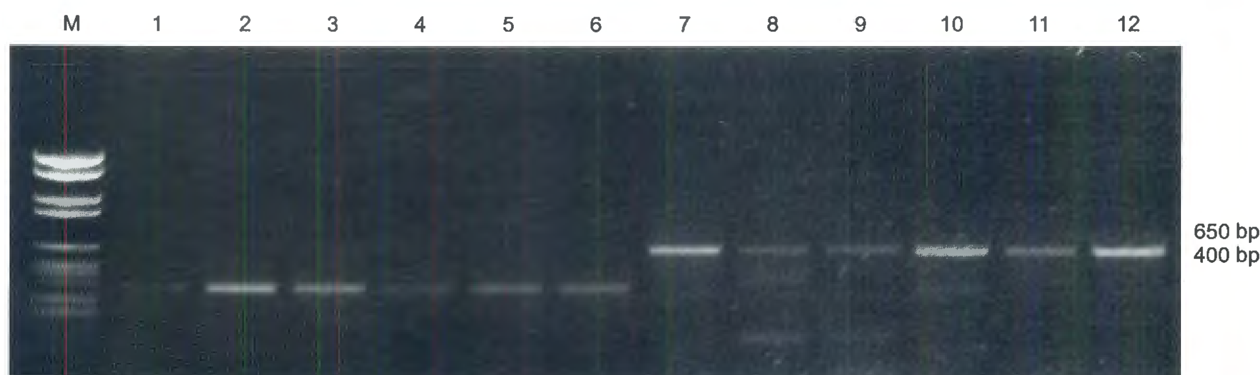

FIG. 2b Demonstration of second round amplicons with the biotype-specific primers P_{viv} (+) and P_{can} (+). G-L PCR products were used as templates in PCR reactions mixtures which each contained P_{viv} (+), P_{can} (+) and L (–) primers. The expected amplification products of 650 bp and 400 bp were obtained for viverrid (lanes 7–12) and canid viruses (lanes 1–6), respectively. A DNA molecular weight marker (marker VI, Boehringer Mannheim) is shown in lane M

between canid and viverrid rabies virus isolates, considering the importance of exact complementarity in the 3' end of primers for successful priming of the PCR.

RNA extraction, first round reverse transcription PCR and subsequent type-specific analysis

A number of isolates of known sequence identity were selected in order to determine whether the newly designed primers differentially amplify viruses specific to the canid or viverrid group. Template for the virus-specific typing was generated by a first round amplification of reverse transcribed viral RNA, using the G-L primer pair and yielded a virus-specific PCR product of approximately 850 bp (Fig. 2a). Second round amplification was carried out in a single reaction containing the P_{can}(+), P_{viv}(+) and L(–) primers and yielded the expected amplicons of 650 bp (viverrid biotype, Fig. 2b) or 400 bp (canid biotype, Fig. 2b). No cross-reactivity of any of the primers occurred on heterologous templates, thus confirming the specificity and reliability of the type-specific primers.

TABLE 2 Oligonucleotide primers used to differentiate between canid and viverrid rabies virus biotypes

Oligonucleotide primer	$P_{can}(+)$	$P_{viv}(+)$
Template specificity	Canid	Viverrid
Sequence	5'-TTC GGG TGA CTC CGT GC-3'	5'-TTC GGG TGA CTC CGT GC-3'
Tm on target template	50 °C	45 °C
Tm on non-target template	18 °C	12 °C

The usefulness of these primers to accurately distinguish between the different viral biotypes was subsequently investigated in an experiment in which 42 additional isolates from various different hosts were subjected to the PCR analysis. No sequence data was available for these isolates, but monoclonal antibody (Mab) typing had indicated that these isolates were either of the canid (18 isolates) or viverrid (24 isolates) biotype (Table 2). PCR amplified cDNA from the genomes of each of these isolates was shown to be consistently and reliably amplified by second round PCR from the expected priming positions and to the expected amplicon size, in accordance to the biotype designation (Mab) of each. A single dominant amplicon of high yield was obtained in all experiments.

DISCUSSION

For confirmation of rabies virus infection, diagnostic tests such as the intracerebral inoculation of newborn mice, tissue culture infection and the rapid rabies enzyme immunodiagnosis represent significant advances made over the last 30 years (Webster & Casey 1988). The major drawback of most of these techniques, especially the intracerebral inoculation, is the amount of time required before a positive confirmation can be made (Sacramento, Bourhy & Tordo 1991). Consequently, less time-consuming serological assays, such as the detection of the virus antigen by immunofluorescence, have become the most widely used diagnostic tools (Bourhy, Tordo, Lafon & Sureau 1989; Swanepoel, 1994). In more recent times, DNA sequencing is increasingly applied to accurately type and characterize viral genomes. However, its application to numerous samples can be impractical even when sequencing PCR products directly.

Rabies viruses isolated in southern Africa have been characterized antigenically (King *et al.* 1993) and it is only recently that comparative sequence analysis was applied as an alternative and augmentative technique, subsequently applied in order to characterize the canid and viverrid virus biotypes of South Africa (Nel *et al.* 1997; Von Teichman *et al.* 1995). Here the development of a competitive hemi-nested PCR assay to rapidly differentiate between the canid and the viverrid viruses was described. Comparative sequence analysis of the G-L intergenic region and cytoplasmic domain of the glycoprotein indicated these domains to be sufficiently variable (33% overall) to allow for the design of oligonucleotides which, when used as primers for PCR, would allow for differentiation between rabies virus biotypes. Thus, the pattern and distribution of nucleotide substitutions between the two biotypes were exploited towards the design of biotype-specific oligonucleotides which would produce characteristic amplicons permitting discrimination of the biotypes solely on the basis of PCR amplicon size. Our strategy was to use primers which are homologous to highly conserved regions on the rabies genome to ensure the amplification of rabies virus genetic material during a first round of amplification, followed by the type-specific PCR assay. The success of the designed primers was assayed in a trial consisting of rabies viruses from diverse origin and host species and were found to consistently and reliably react biotype specific when used in a competitive hemi-nested reaction. In a related investigation the conserved portion of the nucleoprotein was considered for the design of a type-specific primer, given the comparatively larger amounts of nucleoprotein-specific mRNA in infected cells. However, this primer showed a decreased discriminatory ability by cross-reacting with heterologous templates. This result was not entirely unexpected, considering the high degree of conservation within the nucleoprotein genes of rabies virus isolates.

The differentiation of the rabies viruses is important in understanding the viral epidemiology and subsequent development and adjustment of disease control measures. This type-specific assay therefore provides a simple method to diagnose and/or type isolates within 3 h after virus RNA extraction. The technique should be a useful additional tool in virus diagnostics and should especially find application in cases where samples are degraded or where samples were treated with formalin or other preservatives.

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