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Molecular epidemiology of rabies epizootics in Texas

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Abstract

Background: Texas is in the midst of two independent epizootics of rabies, involving coyotes (*Canis latrans*) and domestic dogs (*Canis familiaris*) in southern Texas and grey foxes (*Urocyon cinereoargenteus*) in west central Texas. The domestic dog/coyote (DDC) and grey fox (TF) rabies virus variants cannot be differentiated by antigenic typing with currently available monoclonal antibodies. These two variants also cannot be distinguished from a third variant, Sonora dog (SD) rabies, that is not enzootic in Texas, but occasionally occurs in animals along the western border with Mexico.

Objectives: To determine a method for the differentiation of the DDC, TF and SD variants, which is essential for epidemiologic monitoring of the Oral Rabies Vaccination Program (ORVP), a program instituted to control rabies in coyotes and grey foxes in Texas.

Study Design: Primers complementary to nucleoprotein sequence of either the DDC or TF rabies virus permit specific reverse transcription and amplification by polymerase chain reaction. In addition, general primers, which recognize a broad range of rabies variants, used in conjunction with a restriction digest for the differentiation of DDC, TF or SD rabies virus were investigated.

Results and Conclusions: Of 122 specimens tested with specific primers, 111 (91%) were specifically identified as either DDC (33 samples) or TF (78 samples). Overly stringent conditions, enzyme inhibitors, or limiting RNA may account for the 11 non-amplifications. Amplification of RNA under less stringent conditions, with primers recognizing a broad range of rabies variants followed by digestion with either restriction enzyme *Desulfovibrio desulfuricans I* (*Dde*I) or *Haemophilus influenzae* Rf. (*Hin*fI), was used to identify the 11 isolates that did not amplify with specific primers (6 DDC, 4 TF and 1 SD). In addition to these 11 isolates, the less stringent method of amplification, followed by enzyme digestion has identified a total of 125 additional specimens (26 DDC, 94 TF and 5 SD) that were not tested by variant-specific amplification. These data provide a means to track the spread of the different rabies virus variants and allow the ORVP to plan its vaccine disbursement by defining the two epizootic boundaries. © 1997 Elsevier Science B.V.

Keywords: Molecular epidemiology; Rabies; Rabies epizootic; RT-PCR; Wildlife vaccination

Abbreviations: DDC, domestic dog/coyote; MAb, monoclonal antibody; PCR, polymerase chain reaction; RNA, ribonucleic acid; RT, reverse transcription; SD, Sonora dog; TF, Texas fox.

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1. Introduction

Terrestrial animal rabies in Texas is maintained primarily in three ecotypes, designated as south central skunk, Texas grey fox (TF) and domestic dog/coyote (DDC). Variants are spread primarily by intraspecific transmission within a dominant reservoir, although spillover infection to other species occurs. The virus is also found in insectivorous bats in multiple independent reservoirs of different bat species with distinct virus variants (Bourhy et al., 1992; Smith et al., 1992; Krebs et al., 1995).

In 1988, a canine rabies epizootic began in southern Texas (Starr County, TX). The viral ecotype that previously had been confined to urban dogs in Mexico (DDC) became established in the coyote (*Canis latrans*) population along the US-Republic of Mexico border. The DDC variant is readily transmitted from coyotes to domestic dogs and subsequently, between domestic dogs (Clark et al., 1994). The epizootic spread to 21 contiguous southern Texas counties, where 693 cases of rabies were reported. Concomitantly, in 1988, a second rabies epizootic began in west central Texas (Sutton County, TX) and spread to 48 contiguous counties with 865 cases of rabies reported. This second epizootic is due to the TF rabies variant.

Epidemiologic monitoring of variants is necessary to map the epizootic 'fronts', to specifically target flight patterns for dispersal of recombinant rabies vaccine-laden baits for the two epizootics, to identify translocations of rabid animals and to investigate historical perspectives of the different ecotypes. Identification of most variants can be accomplished by their reaction with panels of monoclonal antibodies (MAbs) (Smith et al., 1986; Smith, 1988, 1989). However, the canid variants associated with the epizootics in Texas and the SD variant are antigenically indistinguishable and are classified by MAb typing as Texas fox-domestic dog/coyote (TFDDC). Thus, in December 1994, the Texas Department of Health Laboratories (Austin, TX) in collaboration with the Rabies Section (Centers for Disease Control and Prevention, Atlanta, GA) began to use genetic typing methods to distinguish the two variants causing the epizootics. The results presented here demonstrate that genetic typing of rabies virus is a powerful investigative tool, essential for epidemiologic studies.

2. Materials and methods

2.1. Specimen selection

Brain tissues were tested by direct immunofluorescence (Centocor, Malvern, PA; BBL Microbiology Systems, Cockeysville, MD) microscopy for rabies antigen (Dean and Abelseth, 1973). A total of 247 positive specimens were characterized initially as TFDDC by their reaction with MAbs against the nucleoprotein of the rabies virus. Monoclonal antibodies were provided from the Centers for Disease Control and Prevention (Atlanta, GA) and have been used extensively to identify variants of rabies virus (Smith et al., 1986; Smith, 1988, 1989).

2.2. Genetic analysis—RNA extraction

Ribonucleic acid (RNA) was obtained from infected brain specimens by extraction with Total RNA Isolation Reagent (TRIzol, Life Technologies, Grand Island, NY), a monophasic solution of phenol and guanidine isothiocyanate (Maniatis et al., 1982, Farrell, 1989; Higuchi, 1989; Carothers et al., 1992). Homogenization of 50–100 mg of tissue and subsequent precipitation of RNA was performed according to the manufacturer's instructions. The precipitated RNA was resuspended in 100 μl of water and vortexed vigorously. Samples were kept on ice for immediate use or stored long term at -80°C.

2.3. Reverse transcription

Reverse transcription was performed by the addition of 5 μl of extracted RNA to 1 μl of 5 μM primer. The samples were heated for 1 min at 94°C and cooled on ice. Samples were then added to a reverse transcriptase reaction mixture at a final volume of 20 μl, containing 148 mM Tris buffer (pH 8.3), 200 mM KCl, 27.4 mM $MgCl_2$,

0.74 mM each dNTP (Promega, Madison, WI), 1.49 mM dithiothreitol, 50.25 units of avian myeblastosis virus reverse transcriptase (Boehringer Mannheim, IN) and 66.8 units of RNAsin (Boehringer Mannheim) and incubated for 90 min at 42°C.

2.4. Variant-specific amplification

Type-specific amplification was performed on 122 TFDDC specimens with primers complementary to nucleotide sequence obtained previously (Smith et al., 1992), for the variants found primarily in dogs/coyotes or foxes. Primers for the RT reaction included a TxCy primer for RT and amplification of the DDC variant, TGG AAC TGT CAA CTC CGA C; and a shFx primer for RT and amplification of the TF variant, ACC GTC AAT TCC GAT. The reverse primer for all type specific amplification was derived from sequence encoding the carboxy terminus of the nucleoprotein (105, TTC TTA TGA GTC ACT CGA ATA TGT CTT GTT TAG). Amplification of the complementary DNA (cDNA) with the polymerase chain reaction (PCR) (Mullis and Faloona, 1987; Higuchi, 1989) was performed by adding an 80 μl reaction mixture containing 100 mM Tris buffer (pH 8.3), 16.1 units of *Thermus aquaticus* (*Taq*) polymerase (Perkin-Elmer Cetus, Norwalk, CT), and 1.25 pmol TxCy or shFX primers and 1.57 pmol primer 105. Samples were denatured for 1 min at 94°C; 40 cycles of denaturation at 92°C for 30 s, annealing at 50°C for 30 s and DNA polymerization (extension) at 72°C for 90 s were repeated in a thermocycler (Perkin-Elmer Cetus). Amplified DNA was visualized by electrophoresis of a 15 μl sample in 4% NuSieve agarose precast gels (FMC BioProducts, Rockland, MD) prepared in 1 × TBE (0.089 M Tris–borate, 0.089 M boric acid and 0.002 M EDTA) containing 0.5 μg/ml ethidium bromide. The expected product of amplification was a 220-bp fragment with the type-specific primer only.

2.5. General amplification

General amplification was performed on 136 specimens, which included 125 specimens not tested by variant-specific analysis and the 11 that did not amplify by variant-specific primers. Reverse transcription of the variants found primarily in dogs/coyotes or foxes was performed with a primer complementary to sequence encoding the amino terminus of the nucleoprotein (10g, CTA CAA TGG ATG CCG AC). Amplification was performed with the primer pair 10g and a reverse primer (304, TTG ACG AAG ATC TTG CTC AT), complementary to sequence encoding the amino terminus of the nonstructural or phosphoprotein. Reverse transcription was performed, as described above, with the substitution of 14.8 mM $MgCl_2$ in the RT mixture. Amplification of cDNA was performed, as described above, with 40 cycles of denaturation at 94°C for 30 s, annealing at 37°C for 30 s, and DNA polymerization at 72°C for 90 s. The expected product of amplification was a 1449-bp fragment.

2.6. Restriction enzyme digestion

A restriction endonuclease digestion was performed on the 136 specimens amplified by the general primer pair, 10g and 304, by the addition of 20 μl of amplicon (10g and 304 product), 10 units of restriction enzyme *Dde*I or *Hin*fI (Boehringer Mannheim), and 2.5 μl of buffer H supplied by the manufacturer. The mixture was incubated at 37°C for 1.5 h. The reaction was stopped by the addition of 0.5 μl of 0.5 M EDTA and the mixture was placed on ice (Maniatis et al., 1982). Fragment patterns were resolved by electrophoresis for the TF variant (*Dde*I: fragments of 603, 467, 287 and 190 bp; *Hin*fI: fragments of 1021, 291, 120 and 46 bp), DDC variant (*Dde*I: fragments of 831, 421, 271 and 64 bp; *Hin*fI: fragments of 719, 291, 121 and 46 bp) and SD variant (*Dde*I: fragments of 479, 414, 301, 271 and 61 bp; *Hin*fI: fragments of 692, 292, 149, 121 and 46 bp) as described above for variant-specific amplification.

3. Results

3.1. Variant-specific primer analysis

Ribonucleic acid extracted from two rabies samples identified by nucleotide sequence analysis

as the TF or DDC rabies variant was reverse transcribed and amplified only by its type specific primer (Fig. 1). Of 122 rabies samples initially identified as TFDDC on the basis of antigenic analysis and geographic locale, 111 (90.9%) were amplified with one of the two type-specific primers (Fig. 2A,B). Eleven samples failed to amplify with either of the two type-specific primers.

3.2. Restriction endonuclease analysis

Ribonucleic acid extracted from the two rabies samples identified by nucleotide sequence analysis as TF or DDC and tested with type specific primers above was reverse transcribed and amplified with the general rabies virus primers, 10g and 304. A PCR product of appropriate size (1449 bp) was formed for these two rabies variants and the 11 samples that failed to amplify with the type specific primers. Enzyme digestion of the purified PCR product with either *Dde*I or *Hin*fI produced unique DNA fragment patterns for both the TF variant and DDC variant (Fig. 3). Ten of the samples that failed to amplify with type-specific primers were identified as TF rabies variant (4) or DDC rabies variant (6) by amplification with the general rabies virus primers and enzyme digestion. The enzyme digestion pattern of the remaining sample was distinct from that of either the TF or DDC variant (Fig. 3). Nucleotide sequence analysis of this sample (coyote, Presidio County, TX) identified the virus as associated with domestic dogs and other animals in western Mexico (SD). The less stringent method of amplification followed by enzyme digestion has also identified an additional 125 specimens (26 DDC, 94 TF and 5 SD) not tested by variant-specific amplification.

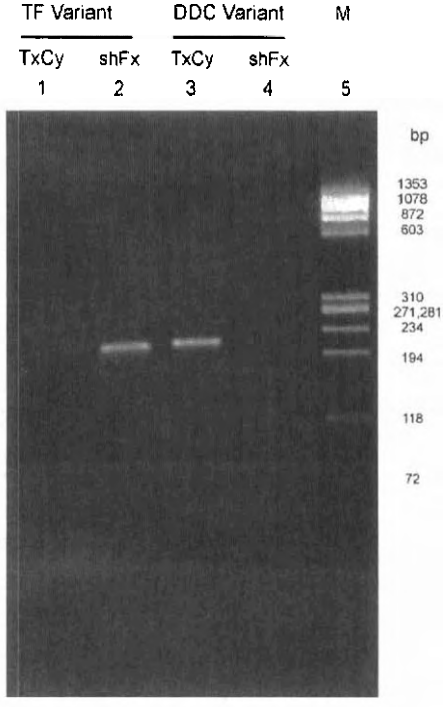

Fig. 1. Ethidium bromide-stained agarose gel electrophoresis analysis of cDNA amplified with primers TxCy:105 (lanes 1, 3) and shFx:105 (lanes 2, 4). Lanes 1 and 2: virus isolate identified by nucleotide sequence analysis as TF rabies variant. Lanes 3 and 4: virus isolate identified by nucleotide sequence analysis as DDC rabies variant. Lane 5: ΦX174/*Hae*III marker fragments.

4. Discussion

Monoclonal antibodies against the nucleoprotein of rabies virus will not differentiate between the TF, DDC and a third variant, SD; that is not enzootic in Texas, but occasionally occurs in animals along the western border of the state with Mexico. Limited sequence analysis of the nucleoprotein gene has determined that the TF and DDC variants in Texas differ from each other at only 11 (5.5%) nucleotide positions (Smith et al., 1992). Because of their homologous nature, it was necessary to distinguish the TF and DDC variants by molecular methods.

Type-specific amplification identified over 90% of the TFDDC specimens tested. Several reasons may explain the failure to identify the remaining 10% of TFDDC specimens, including the necessary high annealing temperature, the $MgCl_2$ concentration, or inhibitors from the original brain tissues (Higuchi, 1989; Mercier et al., 1990, Khan et al., 1991; Wiedbrauk et al., 1995). To complement this technique, amplification with a different primer pair under less stringent conditions, followed by digestion with either restriction enzyme

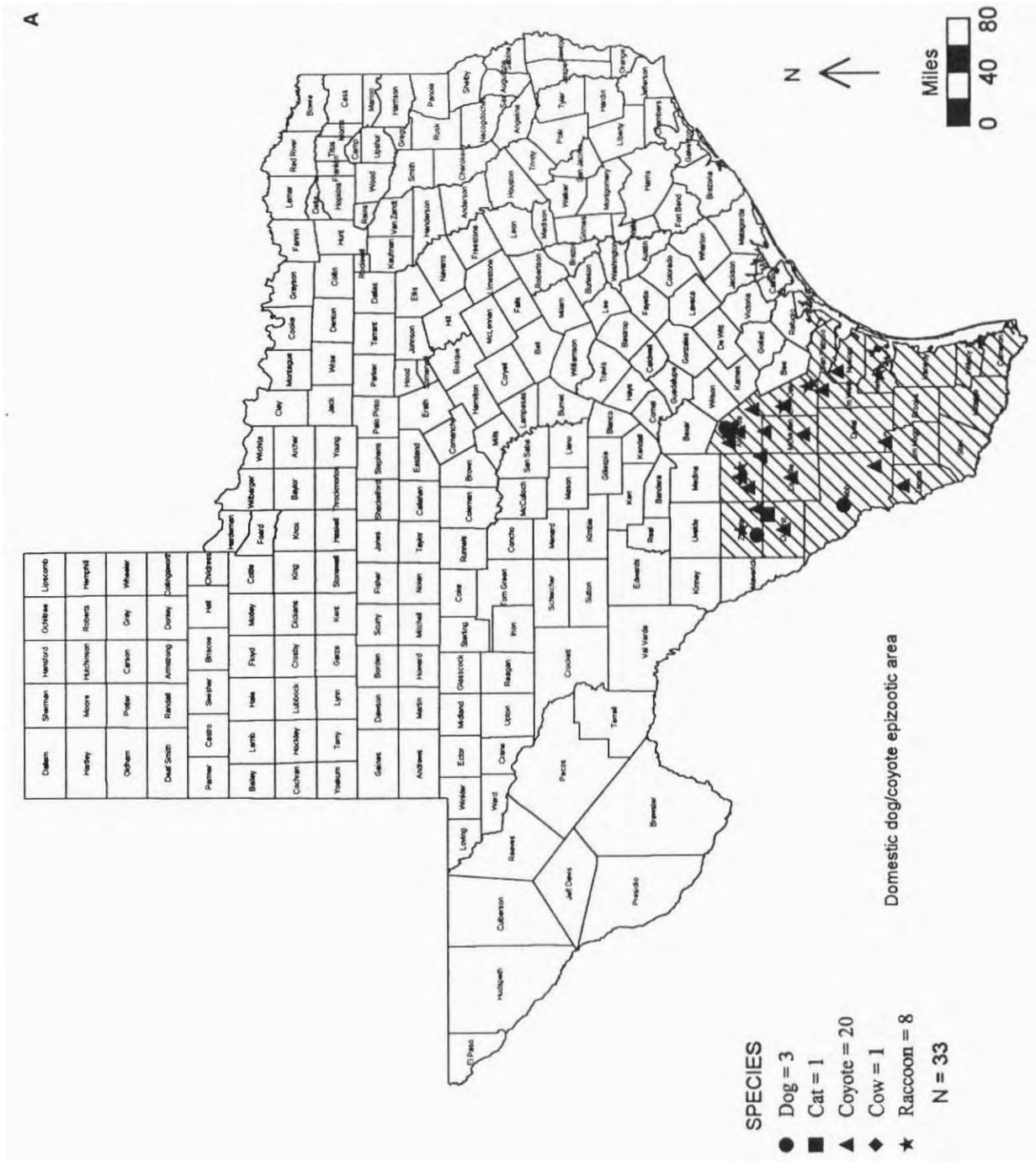

Fig. 2. A. Geographical distribution of rabies cases caused by the domestic dog/coyote (DDC) variant as identified by reverse transcription-polymerase chain reaction specific primers.

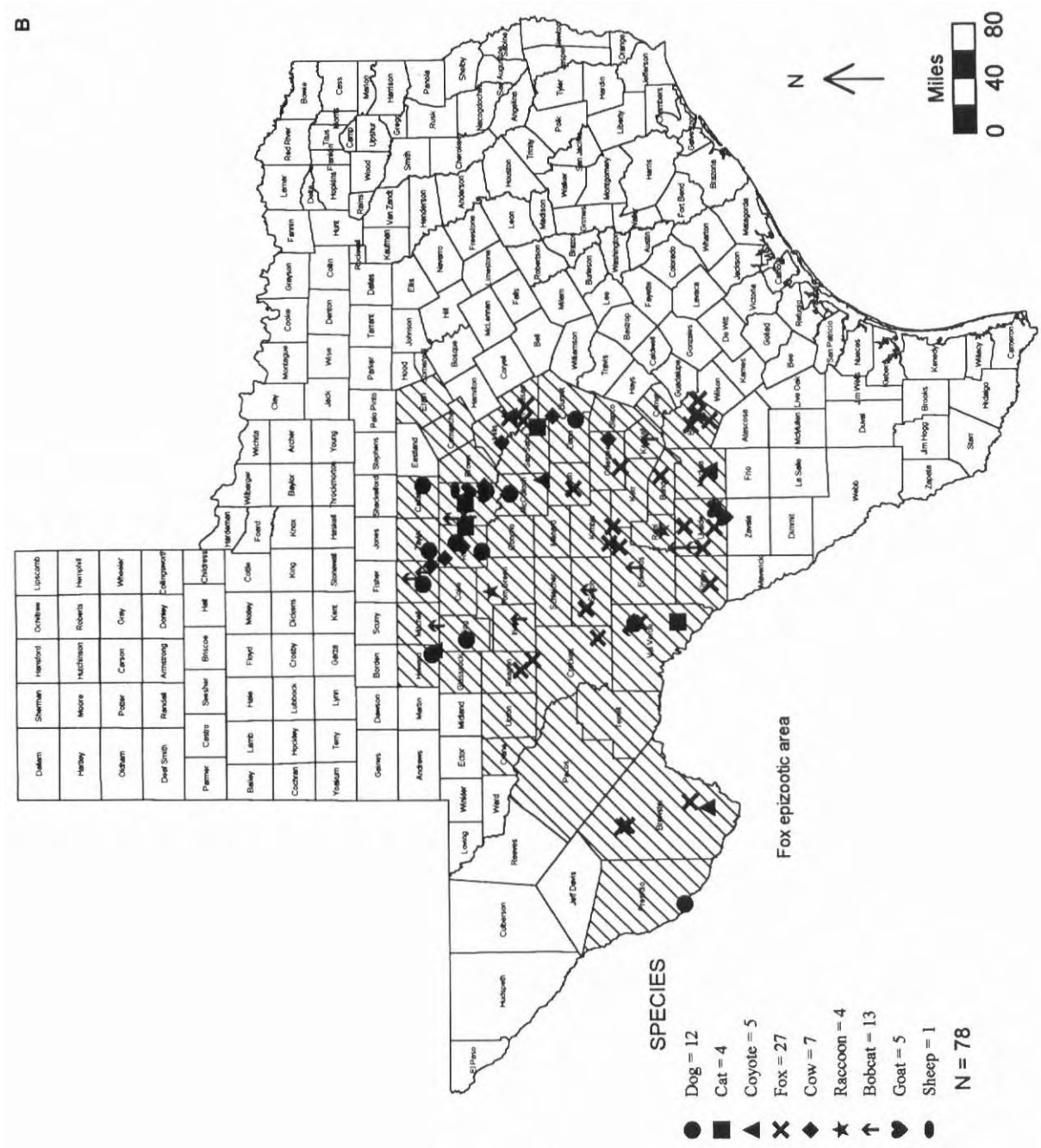

Fig. 2. (*continued*). B. Geographical distribution of rabies cases caused by the grey fox (TF) variant, as identified by reverse transcription-polymerase chain reaction specific primers.

*Dde*I or *Hin*fI was developed. This allowed specimens that could not be amplified by type-specific amplification to be analyzed.

Type specific amplification proved invaluable as a molecular detective to differentiate TF from DDC variants. This method has an advantage of less time for testing ($\sim$2–3 h) because of the direct identification of a variant with the visualization of the PCR product without an enzyme digestion. However, a sacrifice of sensitivity is made due to the necessary stringent conditions, resulting in a lack of amplification for a small number of specimens. General amplification, followed by restriction digest analysis, identified both the 11 specimens not amplified by type-specific amplification and an additional 125 specimens typed as TFDDC by MAb typing. This method has not failed to amplify a rabies specimen positive by direct immunofluorescence. Another advantage of this method is that it permits the identification of other rabies variants that have identical or difficult to interpret MAb patterns. A coyote from Presidio County identified as TFDDC by MAb during the 1995 Oral Rabies Vaccination Program was cause for concern, since the location of the coyote was outside of the vaccine study area. Nucleotide sequence analysis identified the sample as the SD variant, which can be differentiated from TF and DDC by restriction enzyme analysis. The detection of this variant is critical to track the transmission of the virus, to avoid assigning the wrong variant identification, and to help avert a third rabies epizootic. Thus, the primer pair 10g:304, which recognizes a broad range of rabies virus variants and requires less stringent annealing conditions (37°C), and the restriction endonuclease digest, are currently being used to track the different rabies variants.

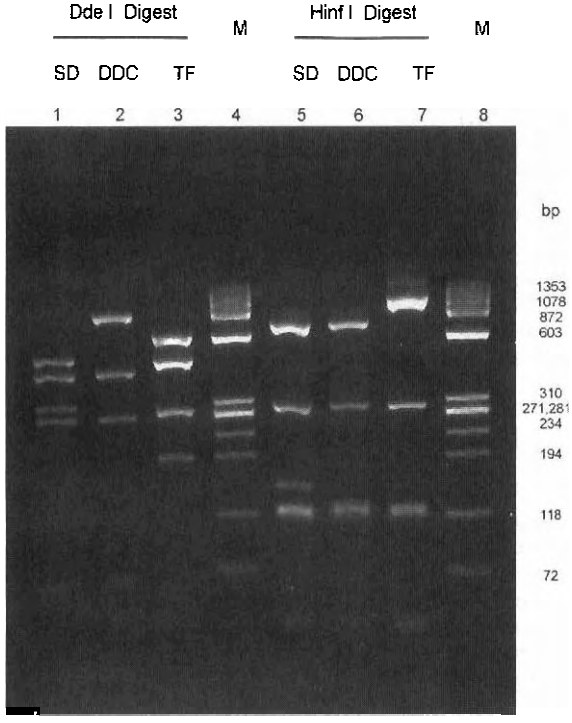

Fig. 3. Ethidium bromide-stained agarose gel electrophoresis analysis of CDNA amplified with primers 10g:304 and digested by either *Dde*I (lanes 1–3) or *Hin*fI (lanes 5–7). Lanes 1 and 5 (SD variant); lanes 2 and 6 (DDC variant); lanes 3 and 7 (TF variant). Lanes 4 and 8 (ΦX174/ *Hae*III marker frag-

Rabies control in wildlife through oral vaccination is being tested in the US (Rupprecht et al., 1986). Texas is in the third year of a 5–7 year Oral Rabies Vaccination Program which was initiated to first contain and then eliminate DDC and TF rabies from the state (Fearneyhough et al., in press). Such programs have been successful in controlling fox rabies in parts of Europe (Brochier et al., 1991) and in Canada (Rosatte et al., 1992). The epidemiologic aspect of molecular typing is important in Texas because control programs must address epizootics to two canid species with overlapping ranges. By identifying the species primarily responsible for transmission of the virus in a certain area, bait design and placement can be more precisely tailored to the specific animal (e.g. larger fish meal baits for dogs/coyotes and smaller dog food baits for grey foxes). It also assists in determining the minimum effective geographic area to bait, frequency of bait application and the time of year for vaccination.

The molecular identification capabilities also provide a method to detect translocations of infected animals within and outside of state borders. Movement of infected coyotes from south Texas is thought to be responsible for the DDC variant's occurring in a hunting dog in Alabama in 1993 (Krebs et al., 1994; Rupprecht et al., 1995) and seven dogs in Florida in 1994 (Centers for Disease

Control and Prevention, 1995; Rupprecht et al., 1995; Krebs et al., 1995). Such translocations also have occurred within Texas. For example, a rabies positive fox specimen from Kleberg County was identified as TF rabies. Kleberg County is in south Texas within the DDC-epizootic boundaries and is 201 km from the TF-epizootic area. Based upon the molecular typing result, an investigation revealed that the fox had originated from Kinney County (TX), which is within the TF-epizootic area.

The successful collaborations between the CDC and Texas rabies laboratories over the last 10 years (Smith et al., 1986, 1991; Clark et al., 1994) have produced the typing data to identify rabies variants common to animal reservoirs in the southwestern US and Mexico and mapped their geographic distribution. The possibility of the TF and DDC variants merging geographically lend significant reason for such discriminatory methods to assist in coordinating the control program in Texas. Molecular characterization of microorganisms is rapidly becoming the method of choice for the study of relationships among different outbreaks of disease. By expanding our antigenic and genetic typing methods, the ORVP and others who survey rabies will be able to recognize when established reservoirs enlarge or invade into new areas or when different animal species become involved in cycles of rabies virus transmission. This information will complement the flow of national surveillance data by increasing state surveillance activities to include molecular typing of virus samples. The powerful concept of molecular epidemiology raises sufficient impetus for continued research into the identification of rabies virus variants.

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