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Journal of Clinical Virology 12 (1999) 9–19

Journal of Clinical Virology

Typing of rabies virus isolates by DNA enzyme immunoassay

Alain Sabouraud [a], Jean S. Smith [a,*], Lillian A. Orciari [a], Carlos de Mattos [a], Cecilia de Mattos [a], Rodney Rohde [b]

[a] *MS G-33 Rabies Laboratory, Viral & Rickettsial Zoonoses Branch, Division of Viral & Rickettsial Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA 30333, USA*
[b] *Texas Department of Health Zoonosis Control Division, 1100 West 49th Street, Austin, TX 78756, USA*

Received 19 August 1998; received in revised form 4 November 1998; accepted 4 November 1998

Abstract

Background: Alternatives to antigenic typing are needed for epidemiologic surveys of the rabies virus associated with translocated coyotes and foxes, especially in areas where a closely related rabies virus is transmitted by striped skunks. **Objectives:** We developed and evaluated two enzyme based typing methods for rabies virus. The products of a reverse transcription-polymerase chain reaction (RT/PCR) of the nucleoprotein gene were hybridized to type specific probes and detected by enzyme assay after immobilization on microtiter plates. **Study design:** We tested RT/PCR products of 27 rabies isolates by two different DNA enzyme immunoassays (DEIA) and evaluated the quality of the results from the corresponding nucleotide sequence of the samples. **Results:** Using a set of two probes, one of the DEIAs correctly identified 26/27 samples as variants of rabies virus associated with either skunks, foxes, or coyotes. The identity of one fox rabies sample was unresolved by this assay. The second DEIA correctly identified 24/27 samples as variants of rabies virus associated with either skunks, foxes, or coyotes. This assay did not resolve the identity of two fox rabies samples, and misidentified one fox rabies sample as a skunk rabies sample. **Conclusions:** DEIA can be used for epidemiologic studies of variants of rabies virus associated with skunks, foxes, and coyotes. Both DEIA methods were effective when typing probes recognized changes at a minimum of two nucleotide positions between variants, but only one assay method was sufficiently stringent to detect a single base pair mismatch. The inherent mutability of RNA viruses must be considered when designing and evaluating typing methods.

Keywords: Rabies virus; RT-PCR; Molecular epidemiology; RNA sequence; Hybridization; Probe

1. Introduction

Several wildlife species are important reservoirs for rabies virus in the United States (Krebs et al.,

* Corresponding author. Tel.: +1-404-639-1050; fax: +1-404-639-1058; e-mail: jss2@cdc.gov.

1386-6532/99/$ - see front matter
PII: S1386-6532(98)00006-7

1997). Virus transmission is primarily intra-specific and once established in an animal population, endemic disease may persist for decades. Affected areas usually expand only gradually, but the introduction of rabies through the translocation of infected animals can result in new outbreaks in areas distant from the endemic area (Rupprecht and Smith, 1994). Private sector transport of wild canids, especially foxes and coyotes, is of particular concern in areas of the United States where recreational hunting of these animals is popular. Stocking of hunting enclosures often involves the interstate movement of animals (Southeastern Association of Fish and Wildlife Agencies, 1997) and on at least two occasions has resulted in an introduction of rabid animals (Centers for Disease Control, 1995). Licensure of oral rabies vaccines for wildlife enables the public health community to respond to new outbreaks of rabies (Rupprecht et al., 1993), but early detection through enhanced case surveillance with virus typing is a vital component of these control efforts.

Early virus typing studies used monoclonal antibodies to identify five antigenic variants of rabies virus in terrestrial animals in the United States (Smith et al., 1986; Smith, 1989) and constructed maps showing the expected distribution of these variants. Antigenic typing with monoclonal antibodies is inexpensive and can be performed quickly on large numbers of samples, but cannot identify all of the epidemiologically important variants of rabies virus in the United States. Most importantly, this typing method cannot be used for surveillance of fox or coyote rabies in areas where a closely related variant is transmitted by skunks (Smith et al., 1986; Rohde et al., 1998). In 1994, at least five and perhaps seven domestic dogs died of rabies after hunting coyotes in a 320 acre fenced enclosure in Alachua County, Florida (Centers for Disease Control, 1995). Because these cases marked the second occurrence of rabies in domestic hunting dogs in the southeastern United States in which the suspected source of infection was translocation of infected coyotes from Texas, the Centers for Disease Control and Prevention called for intensified surveillance for introduced rabies cases in states where residents participate in coyote hunting in enclosures. Although outbreaks of rabies have not been associated with translocation of the variant associated with gray foxes in Texas and spill-over transmission of this variant to domestic canids is infrequent, a rabid gray fox was part of a 1995 shipment of animals from Texas to Montana (Krebs et al., 1996), and specific surveillance for this variant is also desirable.

Because of its sensitivity and specificity, the RNA reverse transcriptase-polymerase chain reaction (RT-PCR) followed by nucleotide sequencing to identify characteristic patterns of nucleotide substitution is the method of choice for genetic typing of rabies (Smith et al., 1995). However, sequence analysis is laborious and costly and is not likely to obtain general use in large scale surveillance activities. Other genetic methods of virus typing, specific PCR primers or digestion of the PCR product with restriction endonucleases, have also been used (Nadin-Davis et al., 1996; Rohde et al., 1998), but these methods are also relatively laborious, especially for large surveys.

We compared two different DNA enzyme immunoassays (DEIAs). DEIA-A (GEN-ETI-K DEIA; Sorin, Sallugia, Italy) is based on hybridization of a PCR product to a variant-specific biotinylated probe immobilized to streptavidin-coated microplates. The formed hybrids are detected by addition of a monoclonal antibody specific for double-stranded DNA and peroxidase labeled protein A (Monteiro et al., 1997). DEIA-B takes advantage of the fact that oligomeric primers can be labeled with biotin without adversely affecting their efficient use in a PCR reaction (Landgraf et al., 1991). Biotin labeled PCR products are hybridized to digoxigenin labeled variant-specific probes. Hybrids immobilized to streptavidin-coated microplates are detected with peroxidase labeled anti-digoxigenin antibody. We evaluated the specificity of both methods using three variant specific probes in tests of 27 rabies samples.

2. Materials and methods

2.1. Specimens

The 27 samples used in this study (Table 1) were from animals submitted for rabies diagnosis and/or typing to public health laboratories. Texas samples A–O were tested at CDC as blind-coded RNA or the PCR amplicons. Iowa samples 1–10 were tested at CDC as blind-coded PCR amplicons. Other samples (identified by number) were from animal tissues submitted to CDC after diagnosis at state public health laboratories in Texas, Florida, Tennessee, Kentucky, and Ohio.

2.2. RNA extraction

RNA contained in approximately 50 μg of brain tissue was obtained by extraction using the TRIzol™ Reagent (Life Technologies, Grand Island, NY) according to instructions of the manufacturer. Precipitation of RNA in the aqueous phase was carried out with addition of isopropanol and centrifugation at 12 000 × *g* for 10 min. After washing with 75% ethanol, the final pellet was dissolved in 0.1 ml diethylpyrocarbonate (DEPC)-treated water.

2.3. Primers and probes

Sequences of primers and probes used in DEIA-A and DEIA-B are given in Table 2. In DEIA-B, a 304 primer biotinylated at the 5'-end was substituted for unlabeled 304 to generate PCR products. Probes were prepared with either biotin or digoxigenin labels. Biotinylation of oligonucleotides was carried out by incorporating DMT-protected Biotin-phosphoramidite (Glenn Research, Sterling, VA) at the 5'-end during standard synthesis on a 394-8 ABI DNAsynthesizer (Applied Biosystems, Foster City, CA). Oligonucleotides to be labeled with digoxigenin at the 5'-end were initially synthesized with a 5' terminal amine group (Aminolink 2, Applied Biosystems). Aminolinked oligonucleotides were mixed with a 10-fold molar excess of digoxigenin-NHS (Boehringer Mannheim, Mannheim, IN), in 0.1 M sodium carbonate buffer pH 9.0. All oligonucleotides were purified by reverse-phase HPLC.

2.4. RT-PCR

A 20-μl RT reaction contained 5 μl of RNA, 5 pmol primer 10g or primer 509, 0.2 mM concentrations of each deoxynucleotide, 14.8 units of RNAse inhibitor (Boehringer Mannheim), and 7.4 units of AMV reverse transcriptase (Boehringer Mannheim) in the buffer supplied by the manufacturer. After incubation at 42°C for 90 min, an 80-μl PCR mixture was added (10 mM Tris–HCl pH 8.3, 2.5 units of Taq polymerase (Perkin–Elmer–Cetus, Norwalk, Conn.), 20 pmol of primer 10g or primer 509 and 25 pmol of primer 304). After 1 min of denaturation at 94°C, 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 were repeated in a thermocycler (Perkin–Elmer–Cetus). Biotinylated PCR prod-

Table 2
Sequence of oligonucleotide primers and probes

Sequence (5' to 3')	Name	Genome Position[a]	Use
CTACAATGGATGCCGAC	10g	66	RT/PCR primer
GAGAAAGAACTTCAAGA	509	1157	RT/PCR primer
TTGACGAAGATCTTGCTCAT	304	1533	PCR primer[b]
AATCATGATGAATGGAGGTC	Generic	1294	Probe
TCGACTAAAGAGATCGCATA	NCSK	1312	Probe
TCGATTGAAGAGATCACATA	TXCY	1312	Probe
CATTTGCCGAATTTTTAAAC	TXFX	1380	Probe

[a] Genome position indicates the 5' nucleotide of the primer in reference to the sequence of SADB19 (Conzelmann et al., 1990).
[b] Primer 304 biotinylated at the 5' end was substituted for unlabeled 304 to generate PCR products for DEIA-B.

Table 1
Absorbance values for PCR products hybridized with four different probes as detected by DEIA-A and DEIA-B[a]

Reservoir	Sample		Generic probe		NCSK probe		TXCY probe		TXFX probe		Variant identified	
	State	ID	DEIA A	DEIA B	DEIA A	DEIA B	DEIA A	DEIA B	DEIA A	DEIA B	DEIA A	DEIA B
Skunk, North Central and Appalachian States	TN	2403	1.30	0.61	1.23	1.05	0.04	0.07	0.02	0.07	SK	SK
	TN	2865	1.22	0.48	1.13	0.76	0.04	0.08	0.02	0.08	SK	SK
	KY	2572	1.29	0.36	1.21	0.65	0.03	0.06	0.02	0.06	SK	SK
	KY	2876	1.18	0.40	0.82	0.63	0.03	0.06	0.04	0.08	SK	SK
	KY	2877	1.32	0.51	1.11	0.97	0.03	0.1	0.03	0.07	SK	SK
	OH	3602	1.20	0.47	0.79	0.20	0.03	0.07	0.03	0.08	SK	SK
	IO	1	1.22	1.14	0.55	1.14	0.04	0.07	0.02	0.07	SK	SK
	IO	7	1.12	1.03	1.11	1.01	0.03	0.07	0.02	0.05	SK	SK
	IO	8	0.59	0.91	0.28	0.80	0.03	0.05	0.02	0.05	SK	SK
	IO	9	1.10	1.09	0.96	1.07	0.03	0.08	0.02	0.05	SK	SK
	IO	10	0.35	0.99	0.19	1.07	0.03	0.07	0.02	0.04	SK	SK
Coyote, Texas	TX	2555	1.30	0.55	0.02	0.06	1.19	0.45	0.02	0.07	CY	CY
	TX	B	1.36	0.78	0.04	0.07	0.75	0.54	0.03	0.07	CY	CY
	TX	E	1.31	0.72	0.04	0.07	0.97	0.49	0.02	0.04	CY	CY
	TX	F	1.23	0.83	0.08	0.08	0.97	0.66	0.03	0.05	CY	CY
	TX	H	1.20	0.83	0.07	0.07	1.00	0.49	0.03	0.05	CY	CY
	TX	I	1.29	1.04	0.04	0.08	0.93	0.58	0.02	0.07	CY	CY
	TX	J	1.38	0.92	0.04	0.07	1.10	0.40	0.02	0.07	CY	CY
	TX	L	1.31	0.70	0.05	0.08	1.01	0.54	0.03	0.04	CY	CY
	FL	2891	1.17	0.57	0.08	0.08	0.74	0.90	0.03	0.06	CY	CY
	FL	2842	1.19	0.89	0.03	0.10	1.10	1.06	0.03	0.06	CY	CY
Fox, Texas	TX	A	0.44	0.24	0.27	0.22	0.03	0.08	1.26	0.53	FX	FX
	TX	G	0.57	0.23	0.50	0.23	0.04	0.07	1.14	0.43	FX	FX
	TX	O	1.20	0.70	0.25	0.18	0.03	0.08	1.18	0.44	FX	FX
	TX	2599	1.35	0.90	0.32	0.50	0.03	0.07	1.28	0.61	FX	FX?
	TX	2603	1.23	0.95	0.25	0.65	0.03	0.05	1.12	0.55	FX	SK?
	TX	2604	1.33	0.62	0.13	0.67	0.04	0.11	0.04	0.05	?	SK

[a] PCR products were derived from samples of rabies virus associated with skunks (SK), coyotes (CY), and foxes (FX).

ucts for DEIA-B were produced by the same procedure, except for the substitution of biotin-labeled 304 primer at the same concentration. No differences in amplification efficiency between the biotin-labeled and the unlabeled primers were reported when the products were run out on an agarose gel stained with ethidium bromide. Because RNA was not available for the Iowa samples, a 2-μl aliquot of PCR products was reamplified for DEIA-B and sequencing. The same reagents were used, omitting the reverse transcriptase and RNAse inhibitor enzymes.

2.5. Sequencing of PCR products

All PCR products were purified using the WizardTM Minipreps DNA purification system (Promega, Madison, WI) and sequenced by using the ABI PRISM DNA Sequencing Kit (Applied Biosystems) according to manufacturer's instructions. Automated fluorescence sequencing was performed with an Applied Biosystems 373 (version 3.0) sequencer. A 300 bp region of the nucleoprotein gene was aligned with the Pileup and Pretty programs in the GCG software package, version 9.1 (Genetics Computer Group, Madison WI).

2.6. DEIA-A

DEIA-A was carried out by the method of Monteiro et al. (Monteiro et al., 1997). Streptavidin-coated microtiter plates (GEN-ETI-K™ DEIA) were incubated for 18–22 h at 2–8°C with a biotinylated probe (25 ng/100 μl for TXCY and NCSK probes and 5 ng/100 μl for generic and TXFX probes). The probes were diluted in TE (Tris 10mM, EDTA 1mM pH 8.0). The well was then washed five times using the wash solution provided by the manufacturer. Amplified products were denatured on a heat block for 3 min at 95°C and then cooled on ice. Twenty μl of each PCR product diluted 1:5 in TE were added to microtiter plate wells containing 100 μl of the hybridization buffer provided by the manufacturer and the plate incubated for 1 h at 50°C in a water-bath. After washing each well five times in wash solution heated to 50°C, 100 μl of the anti-dsDNA antibody was added to each well at a final concentration recommended by the manufacturer. The plates were then incubated for 30 min at 37°C, rinsed four times, and 100 μl/well of horseradish peroxidase-protein A added at a final concentration recommended by the manufacturer. After a final 30 min incubation at room temperature, the plate was washed four times and 100 μl of the chromogen/substrate mixture (tetramethylbenzidine, hydrogen peroxide) was added to each well. The colorimetric reaction was developed for 30 min at room temperature in the dark, stopped by the addition of 200 μl/well of 1 M sulfuric acid, and the absorbance of each well measured at 450/630 nm (Dynatech MR5000). A blank (hybridization buffer-chromogen-blocking reagent) and 2 PCR negative controls were included in each experiment. The cut-off for the reaction (0.18) was calculated by determination of the mean value of two negative controls plus 0.15 as recommended by the manufacturer.

2.7. DEIA-B

Immulon II microtiter plates (Dynatech Laboratories, Chantilly, VA) were coated overnight at 4°C with 200 ng of biotinylated bovine albumin (Sigma, St. Louis, MO) in 100 μl/well of 0.1 M phosphate-buffered saline (PBS), pH 7.2, washed three times with PBS containing 0.15% Tween 20 (PBS-T), and then saturated with 1000 ng of streptavidin (Sigma) in 100 μl/well of PBS with 0.5% gelatin for 30 min at room temperature with shaking. The plates were washed three times with PBS-T and stored at −20°C until use. The biotinylated PCR product (4 μl) was added to a tube containing a digoxigenin-labeled probe (20 ng/200 μl of TE buffer). The mixture was heated at 95°C for 5 min in a thermocycler and allowed to hybridize at 55°C for 30 min. The hybridization mixture was then divided between two wells of streptavidin-coated microtiter plate and incubated at 37°C for 30 min. After four washings with PBS-T, 100 μl of a 1:2000 dilution of peroxidase-labeled anti-digoxigenin Fab fragments (Boehringer Mannheim) in PBS with 0.5% gelatin and 0.15% Tween 20 was then added to each well and incubated for 1 h at 37°C. After four wash-

ings with PBS-T, 100 μl of the chromogenic substrate (BM blue, POD substrate, Boehringer Mannheim) were added to each well. The colorimetric reaction was developed for 30 min at room temperature in the dark, stopped with the addition of 100 μl/well of 1 M sulfuric acid, and absorbance at 450/630 nm measured as above. A blank (hybridization buffer-chromogen-blocking reagent) and two PCR negative controls were included in each experiment. The cut-off for the reaction (0.15) was calculated as the mean absorbance value obtained with six negative samples plus five standard deviations.

3. Results

Based on sequence data available to us at the start of this study, we designed three oligonucleotide probes to be used in different combinations to identify the variants of rabies virus associated with skunks, foxes, and coyotes (Fig. 1). The NCSK probe (100% homology with skunk rabies virus) is the positive control for surveys conducted in areas of the North Central and Appalachian States where skunk rabies is common. A negative reaction is expected when PCR products containing the coyote virus are reacted with the NCSK probe, as the NCSK probe contains three nucleotide changes relative to coyote virus. The NCSK probe contains one nucleotide change relative to the rabies virus found in foxes. Because a single nucleotide mismatch may not yield a negative hybridization signal when PCR products containing the fox virus are reacted with the NCSK probe, a second probe (TXFX) with 100% homology to fox virus is used as a positive control for that virus. The TXFX probe also serves as a negative control for a survey conducted in a skunk rabies endemic area, since it contains three nucleotide changes relative to skunk rabies virus. A survey in areas of Texas endemic for fox rabies uses the TXFX and TXCY probes as positive and negative controls, respectively. Similarly, the TXCY and TXFX probes are used as positive and negative controls in a survey in areas of Texas where the coyote variant of rabies virus is common. In all surveys, unexpected positive or negative reactions are submitted for sequence analysis to identify the variant of rabies virus involved in the infection.

One additional probe was necessary for the comparative analysis presented here, but is optional for routine surveillance. We used a generic probe recognizing sequence conserved among all three variants to calculate an expected value for hybridization with the amplified product. For routine surveillance, the amount of rabies cDNA in the PCR product and the expected adsorbance value for the DEIA is estimated by the amount of staining observed after electrophoresis of the PCR product with molecular weight markers in an agarose gel stained with ethidium bromide.

To test the utility of these probes, we used samples from two state public health laboratories (Iowa and Texas) that use RT/PCR as part of their diagnostic and/or surveillance activities. These samples and additional samples already available at CDC were tested blind-coded by both DEIA methods using the four probes (Table 1). All reactions were confirmed by sequence analysis (Fig. 1).

All samples associated with skunk rabies were identified correctly in both DEIA-A and DEIA-B. All samples hybridized with the NCSK probe, only background values were detectable with the TXCY and TXFX probes, and no potentially confounding mutations were found by sequence analysis of the probed regions (Fig. 1).

All Texas coyote rabies samples tested in this study were identified correctly in both assays. Only background values were detectable with both the NCSK and TXFX probes and all samples hybridized to high specificity with the TXCY probe. No confounding mutations were found by sequence analysis of any of the probed regions (Fig. 1).

Neither assay identified all of the fox rabies samples unambiguously. The DEIA-A correctly identified five fox rabies virus samples (A, G, O, 2599, and 2603) by their diminished hybridization with the NCSK probe and maximum hybridization with the TXFX probe. Texas fox sample 2604 gave the expected negative or very weak hybridization with the NCSK probe, but failed to react with the TXFX probe and therefore, was

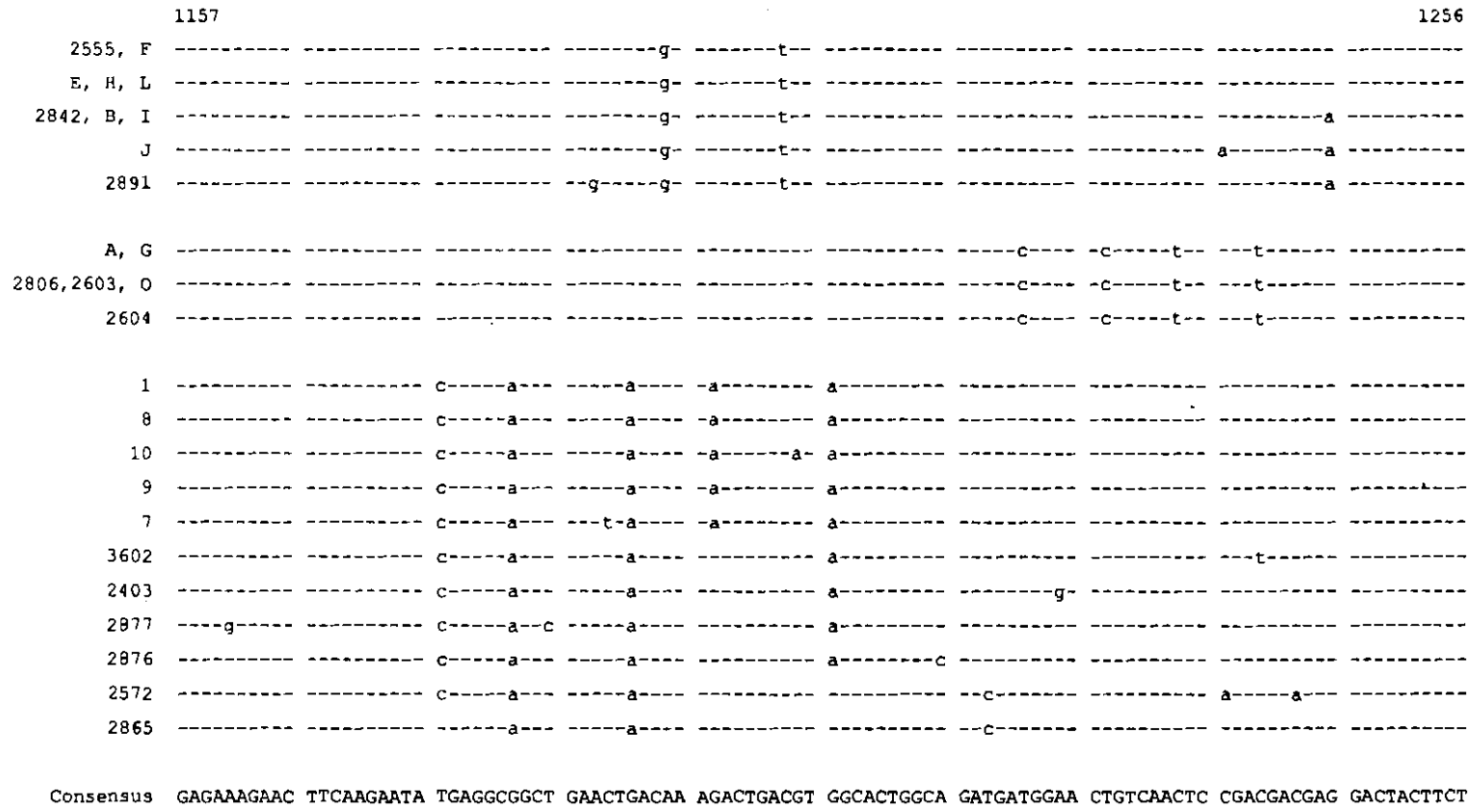

Fig. 1. Nucleotide sequence for rabies samples tested by DEIA-A and DEIA-B (Table 1). Position of probes is indicated by solid line beneath the consensus sequence

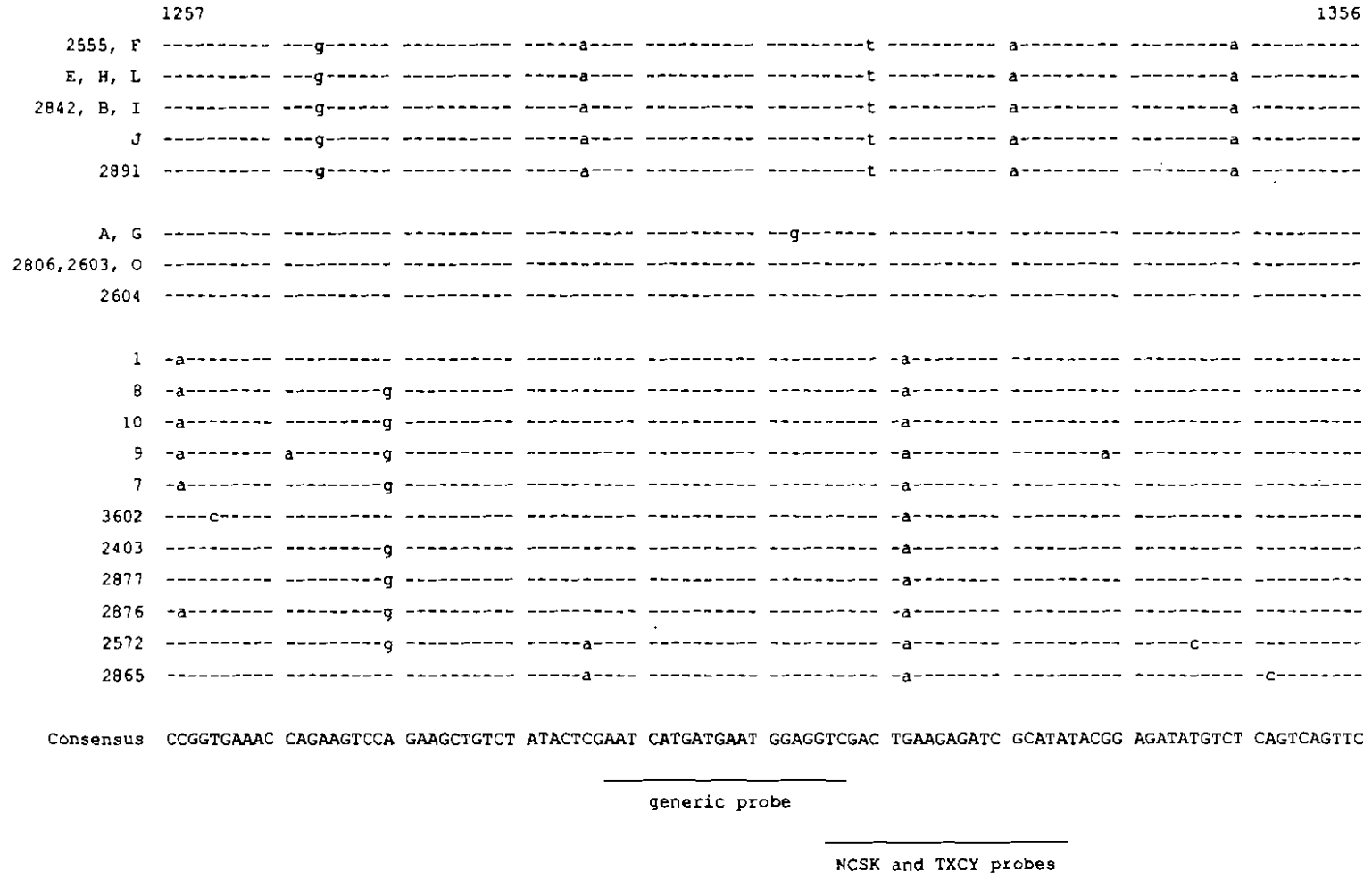

Fig. 1. (*Continued*)

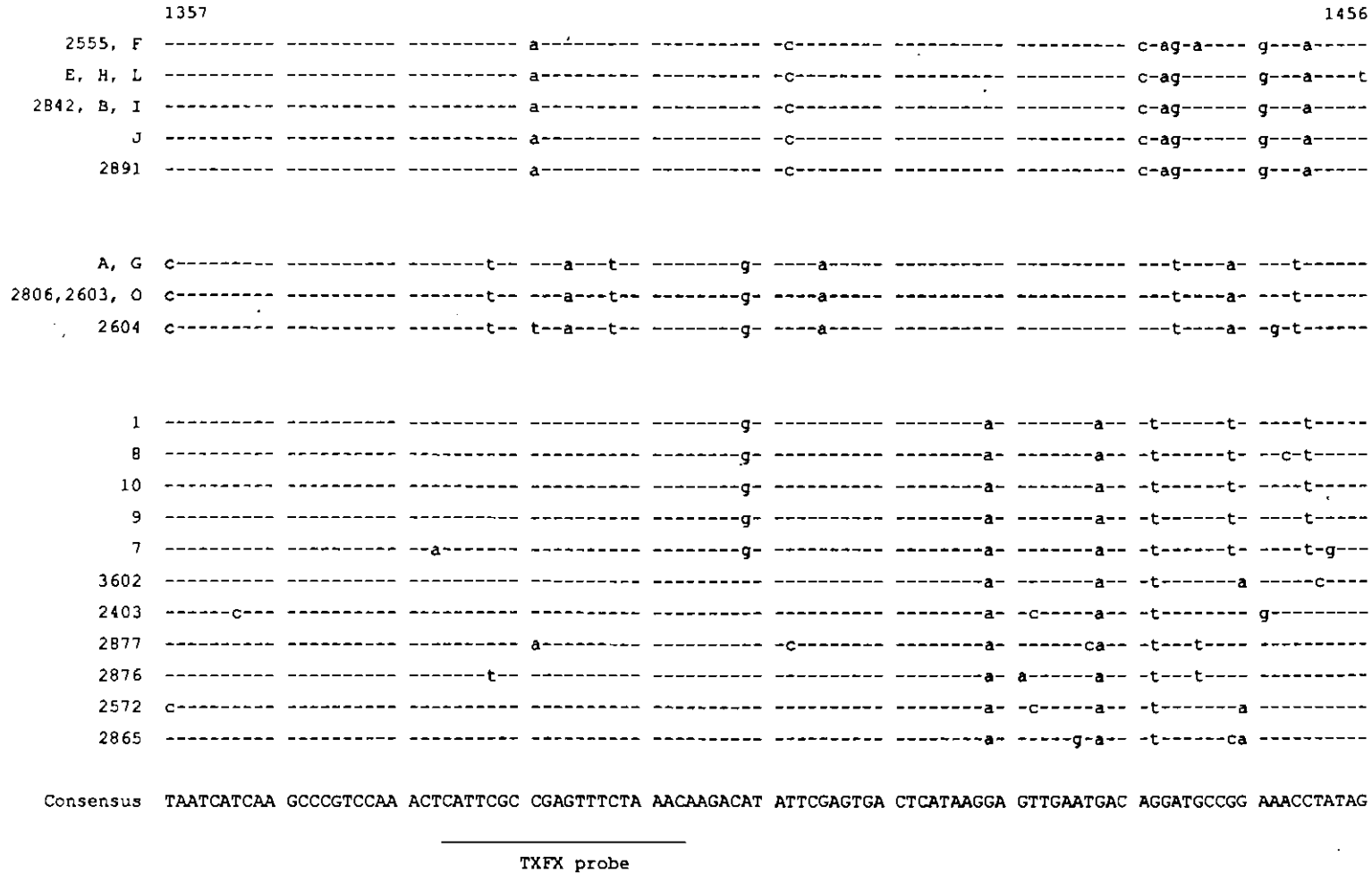

Fig. 1. (*Continued*)

unresolved in the assay. In DEIA-B, hybridization with the NCSK probe was diminished relative to the TXFX probe for four samples; however, because of the lower positive signal with the TXFX probe, the magnitude of the difference between the two probes was less pronounced in DEIA-B as compared to DEIA-A. Also, the NCSK probe in DEIA-B gave a higher signal relative to the TXFX probe for two PCR samples containing fox virus. One of these samples was misidentified as skunk virus because of a negative signal with the TXFX probe. In both assay methods, a lower than expected signal was detected in samples A and G reacted with the generic probe.

Sequence analysis of the Texas fox samples confirmed the expected sequence variation predicted by probe hybridization (Fig. 1). A single nucleotide mismatch in sample 2604 (loss of a G:C bond) completely prevented hybridization by the TXFX probe in both assays. This did not create an misidentification in DEIA-A, since the single mismatch with the NCSK probe (loss of a A:T bond) markedly diminished the hybridization signal. In DEIA-B, however, the single nucleotide mismatch with NCSK probe did not produce a marked reduction in signal and the sample was misidentified. A single nucleotide mismatch in the generic probe region for samples A and G (loss of a A:T bond) also reduced the hybridization signal; however, this probe would not be used in surveys where the expected signal could be determined by estimating the PCR product concentration by comparison against a known standard in an ethidium bromide stained acrylamide gel.

4. Discussion

Because antigenic typing with currently available panels of monoclonal antibodies cannot discriminate between the variant of rabies virus transmitted by striped skunks in the North Central and Appalachian States and the variants transmitted by coyotes and foxes in Texas, nucleotide sequence analysis must be used to survey for translocations or introductions of the canid rabies viruses. Consistent patterns of nucleotide substitution observed in samples from these surveys suggested that a hybridization assay with specific probes could be used for variant identification, thus reducing the number of samples which must be subjected to sequence analysis.

Ideally, a probe should be approximately 20 nucleotides in length and recognize a region of sequence conserved in all samples of a given variant. To avoid false positive hybridizations, the probed region should contain at least two nucleotide positions which vary among the viruses to be discriminated, and because rabies viruses like all RNA viruses are inherently mutable, reaction conditions for the assay should be optimized such that a single nucleotide substitution in the probed region significantly affects hybridization.

Differences in the two assays in the way specific hybrids are formed and hybridization is detected may have contributed to the disparate results obtained by the two methods. In DEIA-A the unlabeled PCR product hybridizes at 50°C to a biotin labeled variant specific probe immobilized on a microtiter plate. This step is followed by washing with a buffer heated to 50°C to increase the stringency of hybridization. Specific hybridization is then revealed with a monoclonal antibody that recognizes only double stranded DNA molecules. In DEIA-B, hybridization of the PCR product and a digoxigenin-labeled probe is in liquid phase at 55°C, but hybridization is followed, not by a wash step as it is in DEIA-A, but by incubation for 1 h at 37°C to permit avidin-biotin capture of the biotinylated PCR product in the microtiter plate well.

Some of the assay conditions could be optimized in both assays in order to obtain the highest signal to noise ratio (data not shown). The concentration of the probes is critical, especially in the DEIA-A where a concentration higher than 25 ng/100 μl resulted in high background OD values. A hybridization temperature below 50°C resulted higher background signal in both assay methods. We tested only 4 μl of each PCR product per well in order to use the original amplicons from the state health laboratories as much as possible; however, it would have been possible to increase the specific hybridization signal in the DEIA-A by adding larger amounts of the PCR product. Increasing the volume of sam-

ple tested in DEIA-B did not increase the OD values. For all reactions in which probe/sample homology was 100%, the OD values were higher in DEIA-A (1.22 with a S.D. of 0.15 with the generic probe) than in DEIA-B (0.76 S.D. 0.22 with the generic probe). Iowa samples 8 and 10 were excluded from this calculation because they were only weakly positive in the initial PCR amplification. Re-amplification of the initial PCR product to produce the biotin labeled product necessary for DEIA-B increased the hybridization signal. For both assays, absorbance values were at background levels with a two nucleotide mismatch between probe and sample. Despite different calculations and lower background in the DEIA-A, cut-off values of both assays were similar (0.18 and 0.15 for DEIA-A and DEIA-B respectively).

The data presented here illustrate some of the drawbacks of probe based typing methods for inherently mutable RNA viruses, but suggest that the methods can be adapted to epidemiologic surveillance of rabies virus. These types of tests are finding increased use in diagnostic laboratories for detection and typing of viruses, bacteria, and other agents (Monteiro et al., 1997). The methods are simple to use, rapid (4–5 h) and require only commonly available equipment. The procedure can be automated, potentially permitting the processing of larger number of samples. The work presented here addressed only three variants of rabies virus, but probes can be designed to fit many different situations. Laboratories using RT/PCR as part of their diagnostic testing for rabies might consider designing probes specific for the variant of rabies virus predominant in their animal population, thus permitting virus typing and confirmatory testing to be performed together.

Acknowledgements

The authors wish to express their thanks to Karla Long and staff of the University of Iowa Hygienic Laboratory and Dr Pushker Raj and staff of the Rabies-Arbovirus Section, Texas Department of Health for their contributions to this study.

References

Centers for Disease Control. Translocation of coyote rabies – Florida, 1994. Morbidity and Mortality Weekly Report 1995;44:580–7.

Conzelmann KK, Cox JH, Schneider LG, Thiel HJ. Molecular cloning and complete nucleotide sequence of the attenuated rabies virus SAD B19. Virology 1990;175:485–99.

Krebs JW, Strine TW, Smith JS, Noah DL, Rupprecht CE, Childs JE. Rabies surveillance in the United States during 1995. J Am Vet Med Assoc 1996;209:2031–44.

Krebs JW, Smith JS, Rupprecht CE, Childs JE. Public veterinary medicine: public health: rabies surveillance in the United States during 1996. J Am Vet Med Assoc 1997;211:1525–39.

Landgraf A, Reckmann B, Pingoud A. Quantitative analysis of polymerase chain reaction (PCR) products using primers labeled with biotin and a fluorescent dye. Anal Biochem 1991;193:231–5.

Monteiro L, Cabrita J, Megraud F. Evaluation of performances of three DNA enzyme immunoassays for detection of Helicobacter pylori PCR products from biopsy specimens. J Clin Microbiol 1997;35:2931–6.

Nadin-Davis SA, Huang W, Wandeler AI. The design of strain-specific polymerase chain reactions for discrimination of the raccoon rabies virus strain from indigenous rabies viruses of Ontario. J Virol Methods 1996;57:1–14.

Rohde, R.E., Neill, S.U., Clark, K.A., Smith, J.S., 1997. Molecular epidemiology of rabies epizootics in Texas, J. Clin. Microbiol. 1997;8:209–17.

Rupprecht CE, Smith JS. Raccoon rabies-the re-emergence of an epizootic in a densely populated area. Semin Virol 1994;5:155–64.

Rupprecht CE, Hanlon CA, Niezgoda M, Buchanan JR, Diehl D, Koprowski H. Recombinant rabies vaccines: efficacy assessment in free-ranging animals. Onderstepoort J Vet Res 1993;60:463–8.

Smith JS. Rabies virus epitopic variation: use in ecologic studies. Adv Virus Res 1989;36:215–53.

Smith JS, Reid-Sanden FL, Roumillat LF, Trimarchi C, Clark K, Baer GM, Winkler WG. Demonstration of antigenic variation among rabies virus isolates by using monoclonal antibodies to nucleocapsid proteins. J Clin Microbiol 1986;24:573–80.

Smith JS, Orciari LA, Yager PA. Molecular epidemiology of rabies in the United States. Semin Virol 1995;6:387–400

Southeastern Association of Fish and Wildlife Agencies, 1997 A survey of fox and coyote running enclosures in Southeastern States. 63 pp.