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Rabies Virus in the Decomposed Brain of an Ethiopian Wolf Detected by Nested Reverse Transcription-Polymerase Chain Reaction

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ABSTRACT: Approximately 75 individuals from a population of 111 Ethiopian wolves (*Canis simensis*) died or disappeared from the Bale Mountains National Park (Ethiopia) between 1988 and 1992 during two significant population declines. Confirmation of rabies virus in two carcasses was based on the fluorescent antibody test (FAT) and the mouse inoculation test (MIT). In an Ethiopian wolf brain previously designated rabies negative by both FAT and MIT, rabies virus was identified by nested reverse transcription-polymerase chain reaction (RT-PCR) and confirmed by Southern blot hybridization. These methods were successfully used on a highly decomposed brain sample which had been stored in 20% dimethyl sulfoxide. This test system allows early and sensitive detection to be undertaken to more effectively prevent spread of disease and thus protect surviving animals.

Key words: *Canis simensis*, case report, Ethiopian wolf, rabies, Southern blot hybridization, reverse transcription-polymerase chain reaction.

The Ethiopian wolf or simien jackal (*Canis simensis*) is an endangered canid, endemic to the Ethiopian highlands. It is the rarest canid in the world, with fewer than 500 surviving animals (Sillero-Zubiri and Gottelli, 1994) and is vulnerable to severe loss or local extinction from disease or other stochastic events.

Rabies is widely reported in domestic dogs throughout Ethiopia (Fekadu, 1982; Mebatsion et al., 1992) and the Ethiopian wolf is susceptible to the existing rabies serotype (serotype 1, classical rabies) and canid strain carried by these dogs. Recently, the existence of two separate disease epizootics was reported in the Bale Mountains National Park (Ethiopia, 6°50′N, 37°10′E) (Sillero-Zubiri et al., 1996). Between 1988 and 1992, 21 male and 19 female wolves died in a population of 111 known animals; an additional 35 wolves were unaccounted for and probably also died (Sillero-Zubiri et al., 1996). The latter epizootic, in the Web Valley (Ethiopia, 7°00′N, 39°40′E) between November 1991 and February 1992, led to the death or disappearance of 41 of 53 adult or sub-adult Ethiopian wolves in five adjacent packs. Two cases of rabies were confirmed in two adult males which died in December 1991, using the fluorescent antibody test (FAT) (Dean and Abelseth, 1973) and the mouse inoculation test (MIT) (Koprowski, 1973).

FAT is the test most usually performed for rabies virus detection and is based upon the microscopic examination of impression smears of brain after treatment with anti-rabies globulin conjugated with fluorescein isothiocyanate. The MIT effects virus isolation through the inoculation of mice by the intracerebral route with a suspension of the test material.

Brain tissue collected earlier from a third wolf in the Web Valley had previously tested negative using FAT and MIT methodologies as previously described in (Sillero-Zubiri et al., 1996). An adult female had died in November 1991, during late pregnancy, <3 km from the males found in December. Clinical signs of hind limb incoordination progressed to complete ataxia and death within 1 wk. A field necropsy did not yield a definitive diagnosis. Reverse transcription (RT) and nested amplification by the polymerase chain reaction (nested RT-PCR) was performed on a sample of this brain and results were confirmed by Southern blot hybridization as described below.

The wolf brain had been stored in 20%

dimethyl sulfoxide (DMSO) due to the unavailability of cooling facilities in the field at the time. DMSO is inhibitory to Taq polymerase, the key enzyme in PCR (Gelfand, 1989). Thus, viral RNA (vRNA) extractions were performed to minimize the presence of this solvent. Total RNA from approximately 2 g of brain was extracted using Tri Reagent (Sigma-Aldrich Company Limited, Poole, Dorset, United Kingdom) according to the manufacturer's instructions but including one additional isopropanol precipitation step and one additional 75% ethanol wash to facilitate more complete removal of DMSO.

Reverse transcription of the vRNA was performed on 2 μg of total brain RNA. The RNA was denatured at 100 C for 5 min, cooled on ice and then added to a final volume of 10 μl containing single strength MMLV-RT buffer (Gibco BRL Life Technologies Limited, Paisley, United Kingdom), 1 mM each dNTP (Promega Limited, Southampton, United Kingdom), 14 units RNAasin (Promega Limited), 1 mM dithiothreitol (Promega Limited), 15 *p*M of messenger sense primer N1 (Tordo et al., 1986) and 200 units of MMLV-RT (Gibco BRL Life Technologies Limited). Reverse transcription was conducted for 60 min at 42 C and on its completion, the reaction mix was boiled for 5 min and then placed on ice. A 10-fold dilution of the reaction mixture was then performed in RNAase free water (Amersham International, Buckinghamshire, United Kingdom). A positive control, 2 μg of cellular RNA extracted from challenge virus standard (CVS) infected brain tissue, and a negative control, 2 μg of total cellular RNA extracted from uninfected brain tissue, were included.

Ten μl of the diluted cDNA-RNA hybrid was amplified in a final volume of 100 μl of a PCR solution containing single strength PCR buffer minus magnesium (Perkin Elmer Cetus, Cheshire, United Kingdom), 200 mM each dNTP, 15 *p*M of each of primers N1 and N4 (Tordo et al., 1986), 2.5 units of Taq polymerase (Perkin Elmer Cetus) and two Hot-Wax-1.5 mM magnesium beads (Invitrogen, R&D Systems Europe Limited, Abingdon, United Kingdom). The amplification was performed on a Perkin Elmer Cetus Thermal Cycler for 5 cycles: denaturation occurred at 95 C for 90 sec, annealing occurred at 45 C for 90 sec, with a pause at 50 C for 20 sec, and elongation occurred at 72 C for 90 sec. This was followed by 30 cycles: (denaturation occurred at 95 C for 30 sec, annealing occurred at 45 C for 60 sec, with a pause at 50 C for 20 sec, and elongation occurred at 72 C for 60 sec). Finally a single cycle was performed: (denaturation occurred at 95 C for 30 sec, annealing occurred at 45 C for 90 sec, with a pause of 50 C for 20 sec, elongation occurred at 72 C for 10 min). The Ethiopian wolf brain total RNA sample was tested in triplicate.

The primary amplification was followed by nested PCR using 1 μl of the primary amplified product which was added to 49 μl of PCR solution, as described for primary PCR, using internal primers JW4 (5′-AGAATGTTTGAGCCACGGCA-3′) and JW5 (5′-TCAGGTGAAACCAGAAGTCC-3′). The amplification was performed using one cycle (denaturation occurred at 95 C for 120 sec), 25 cycles (denaturation occurred at 95 C for 60 sec, annealing occurred at 52 C for 60 sec, elongation occurred at 72 C for 60 sec) and one cycle (elongation occurred at 72 C for 10 min). Twenty μl aliquots of the resultant PCR products were analysed on 1.8% agarose/tris-acetate-EDTA gel, containing ethidium bromide (Sigma-Aldrich Company Limited) and photographed under UV light.

Southern blot hybridization using non-radioactive labelling with digoxigenin-labelled deoxyuridine triphosphate was performed on the amplification product from the first round complementary DNA (cDNA) according to manufacturer's (Boehringer Mannheim UK Limited, East Sussex, United Kingdom) instructions. The probe was amplified by PCR from the CVS rabies virus according to manufactur-

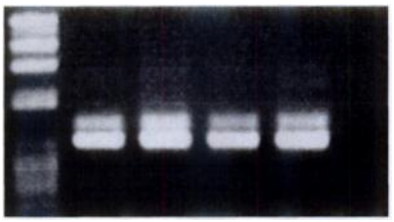

FIGURE 1. Nested RT-PCR of Ethiopian wolf vRNA: Lane 1 is the ϕX174/Hae III marker (Gibco BRL Life Technologies Limited); Lanes 2–4 are the Ethiopian wolf cDNA fragment (396 bp); Lane 5 is the CVS 11 (positive control) cDNA fragment; and Lane 6 is from an uninfected brain (negative control).

er's instructions (Boehringer Mannheim UK Limited) using primers JW4 and JW5.

Based on the nested RT-PCR there was an amplified fragment of cDNA of the predicted size of 396 base pairs (bp) in (Fig. 1). Results from the Southern blot hybridization (Fig. 2) supported the specificity of this result by hybridization of an internal probe to the first round PCR cDNA product (782 bp).

Although the brain sample was in an advanced state of decomposition it was still possible to amplify a region of 782 bp from the vRNA. In addition, the method of vRNA extraction adopted has been successful in removing sufficient levels of the DMSO from the RNA to allow the Taq polymerase to successfully amplify the fragment of cDNA. Thus, nested RT-PCR of short regions of vRNA was shown, in this instance, to have a higher level of sensitivity than the FAT and MIT. In addition, using nested RT-PCR, we were able to detect rabies virus in brain tissue which had undergone extensive decomposition.

Clearly the performance of this test requires particular skills, laboratory facilities and equipment which may not always be available in developing areas. In addition, stringent procedures need to be employed to ensure that false positive results do not result from cross contamination. However, when the situation demands, it may be possible for the test to be performed either by the host country or in collaboration with an appropriately equipped and staffed laboratory elsewhere. In addition, further work has now made additional primers available which can detect all the currently recognised genotypes (1–6) of rabies and rabies-related viruses. This will further enhance the usefulness of this methodology.

This test should prove to be a useful tool in future management of Ethiopian wolves and other endangered wildlife populations

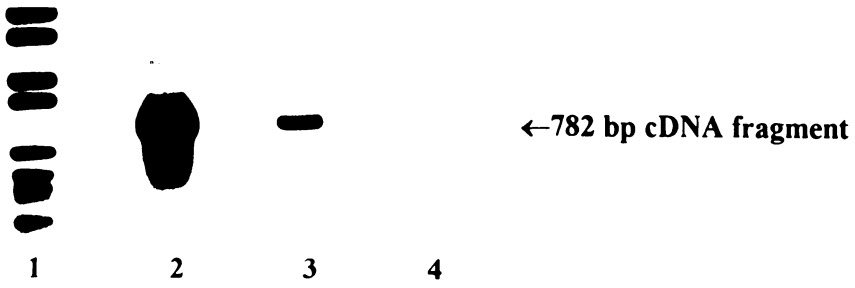

FIGURE 2. Southern blot hybridization of first round RT-PCR of Ethiopian wolf vRNA: Lane 1 is Dig labelled molecular weight markers VI, pBR328/BglI+HinfI (Boehringer, Mannheim, UK; Diagnostics and Biochemicals); Lane 2 is the CVS 11 (positive control) cDNA fragment; Lane 3 is from the Ethiopian wolf cDNA fragment (782 bp); and Lane 4 is from an uninfected brain (negative control).

in more accurately diagnosing cases of rabies, particularly in conditions where there was delay in recovering carcasses. Early and accurate diagnosis allows prompt measures to be undertaken to more effectively prevent spread of disease and thus protect surviving animals.

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