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RABIES EMERGENCE AMONG FOXES IN TURKEY

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ABSTRACT: Sixteen rabies isolates recently collected from mainland Turkey and two isolates held within a British archive were used to form a representative cohort from a range of vectors, and were analyzed to identify potential causes for an increase of rabies within the fox (*Vulpes vulpes*) population in Turkey. Each isolate was characterized by sequence analysis of the nucleoprotein gene and compared phylogenetically to the cohort, to isolates from neighboring countries and to isolates from continental Europe and Russia. From this analysis the isolates could be divided into three groups associated with geographic location. This included a western group, an eastern group, and one isolate that did not group with any other Turkish isolate. This observation was also found using the heteroduplex mobility assay as an alternative method for typing rabies virus isolates. Further comparison with isolates from neighboring countries suggests that this isolate was related to viruses present in Georgia and could represent a recent import to Turkey from that country. Within the two larger groups, sequence data were obtained from both infected dogs and foxes suggesting that there has been transmission of virus between these two species. The direction of transmission could not be identified by the phylogenetic analysis, although absence of rabies within the fox population in previous years suggests that this could represent a recent spillover from the domestic dog to the fox.

Key words: Fox, heteroduplex mobility assay, phylogenetics, rabies, Turkey.

INTRODUCTION

Classical rabies virus (RABV) is transmitted in the saliva of an infected animal to a susceptible host through bite wounds. Following infection, there is a variable incubation period followed by fatal encephalitis. The virus persists through continuous rounds of infection of free-roaming carnivores. In many parts of the world the domestic dog is the principal vector that brings the virus in contact with humans and as such, is the main target for control and eradication of rabies. However, spillover from one carnivore species to another has been documented (Aubert, 1992) and may be a common occurrence, especially during periods of high reproductive rates in a particular species. This presents a challenge to epidemiologists tracking the disease in new populations. Molecular epidemiology offers an approach to investigating the spread of RABV (Bourhy et al., 1999), the existence of rabies within multiple hosts (von Teichmann et al., 1995; de Mattos et al., 2000), and is suited to studying the emergence of rabies in new hosts.

Rabies within Turkey is principally a disease of domestic dogs with occasional cases observed in domestic cattle (World Health Organization, 1998; Aylan and Vos, 2000). Until the late 1990s the incidence of dog rabies was in decline. This trend has reversed in recent years and has coincided with the emergence of rabies within the fox (*Vulpes vulpes*) population (Muller, 2000). It is uncertain at this stage whether this is due to transmission from domestic dogs and establishment of an endemic cycle within the wildlife population. Alternatively, rabies could have been endemic within foxes throughout recent decades and is only now being detected. Studies on the molecular epidemiology of rabies within southeastern Europe and the Middle East have been limited to large global surveys (Smith et al., 1992; Kissi et al., 1995) and a study investigating rabies within Israel (David et al., 2000). This latter work, like many epidemiologic studies of rabies, used sequence data derived from the nucleoprotein-coding region. In Israel, three hosts support rabies (dog, fox, and

jackal [*Canis aureus*]), but the principal host is the fox. In addition, rabies diversity varied with geographic location rather than host, suggesting that virus was readily transmitted between different canid groups. Epidemiologic evidence was presented for linking local isolates with those from Saudi Arabia in the south and Iran to the north. Currently, little is known on the relationship of RABV isolates to each other or to host species within Turkey. There is also little information on the links between rabies in Turkey and RABV isolates from neighboring regions. To address this issue, a cohort of RABV isolates was obtained with the objective of establishing a phylogeny of RABV within Turkey and to use this to understand the emergence of rabies in foxes. This in turn could assist in the development of effective control measures.

The cohort represents RABV isolated from both dogs and foxes in areas of Turkey where rabies is endemic. The RNA was isolated for each RABV and sequence was amplified from the N-terminal of the nucleoprotein-coding region. The sequences derived were compared to other RABV isolates from Turkey and with RABV sequences from countries in close proximity. This approach established an initial phylogeny that was used to test the heteroduplex mobility assay (HMA). Previously, this assay has been used extensively to measure variation of RNA viruses, mixed populations of hepatitis C virus (White et al., 2000), and typing of human immunodeficiency virus 1 isolates (Agwale et al., 2001). However, this study is the first reported use of this technique to type RABV isolates.

METHODS AND MATERIALS

Sixteen RABV isolates were obtained from the brains of infected dogs and foxes submitted to the Etlik Central Veterinary Control and Research Institute (Ankara, Turkey) during 2000 and 2001. Two RABV isolates were included from the Veterinary Laboratory Agency (Weybridge, Addlestone, Surrey, UK) archive. A further five RABV isolates were included from the archive, representing provisional European groups. All RABV material was derived directly from original infected brain material or from RABV passaged in mice. Further RABV nucleoprotein sequences were obtained from GenBank (2002).

Total RNA from infected brain was extracted using the TRIzol method (Life Technologies Inc., Gaithersburg, Maryland, USA) following the manufacturer's instructions and the RNA was then diluted in HPLC grade water (Aldrich, Dorset, UK) to a concentration of 1 μg/μl and stored at −80 C. Reverse transcription was carried out using the primer JW12, which initiates transcription at the 5′ end of the RABV nucleoprotein, as described previously (Heaton et al., 1997). The polymerase chain reaction (PCR) was then used to amplify nucleoprotein sequences with the primers JW12 and a combination of three primers (JW6 DPL, JW6 E, and JW6 M) that amplify all known lyssavirus nucleoprotein sequences (Heaton et al., 1997). An aliquot of the amplification reaction was analyzed by agarose gel electrophoresis and positive bands of 606 base pairs (bp) in size were extracted and purified using the QIAquickTM Gel Extraction Kit (Qiagen, Crawley, UK) following the manufacturers instructions. Direct sequencing reactions were performed using the flanking primers JW12 and JW6 DPL at a concentration of 1pmole/μl. The BigDye™ sequencing kit (Applied Biosystems, Warrington, UK) was used following the manufacturer's protocols. Sequencing reactions were analyzed by the toxicology laboratory at Leicester University (Leicester, UK).

Nucleotide sequences were orientated and edited using the DNAstar program (DNAstar Inc., Madison, Wisconsin). Sequence alignment was carried out using the ClustalW program (Thomson et al., 1994) and transition/transversion ratios estimated by the Puzzle 32 program (Strimmer and von Haeseler, 1996). The neighbor joining method (Saitou and Nei, 1987) was used to construct phylogenetic trees using the PHYLIP 3.5 package (Felsenstein, 1989). Confidence limits were obtained by bootstrap resampling of 100 replicates using the Seqboot, DNAdist and Neighbor programs within PHYLIP. Consensus trees were obtained with the Consense program and generated with the Drawtree program. Bootstrap values were visualized with the Treeview program (Page, 1996) and values >70 were considered significant. The final tree formats were edited using Freelance Graphics (Lotus, IBM Software Group, Cambridge, Massachusetts, USA).

The HMA was used as an alternative technique to create phylogenetic separation of samples. This technique anneals the PCR product

TABLE 1. Turkish rabies virus isolates used for limited sequence analysis of the nucleoprotein-coding region.

Isolate reference	Turkish province	Host	Date isolated	GenBank reference
RV202	Samsun	Dog	1989	AY091608
RV203	Samsun	Wolf	1989	AY091609
RV1124	Manisa	Fox	2000	AY091610
RV1125	Izmir	Fox	2001	AY091611
RV1126	Izmir	Fox	2001	AY091612
RV1127	Manisa	Fox	2001	AY091613
RV1128	Manisa	Fox	2001	AY091614
RV1129	Erzurum	Fox	2001	AY091615
RV1130	Erzurum	Dog	2001	AY091616
RV1131	Gaziantep	Bovine	2001	AY091617
RV1132	Mardin	Bovine	2001	AY091618
RV1133	Ardahan	Dog	2001	AY091619
RV1134	Ardahan	Dog	2001	AY091620
RV1136	Bursa	Dog	2001	AY091621
RV1137	Bursa	Dog	2001	AY091622
RV1140	Istanbul	Dog	2001	AY091623
RV1141	Istanbul	Dog	2001	AY091624
RV1143	Istanbul	Dog	2001	AY091625

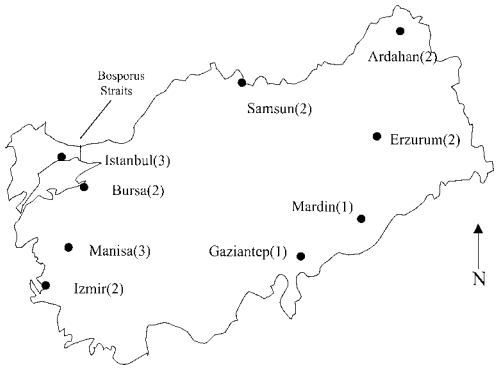

FIGURE 1. Map of Turkey showing the major centers where isolates were obtained. Numbers in brackets indicate the number of isolates from each location.

derived from two isolates and then assesses the change in mobility following separation in a polyacrylamide gel. The greater the difference between the two products the more the heteroduplex will be retarded within the gel. Therefore any change in the mobility of the heteroduplex compared to the homoduplex indicates that two sequences differ in proportion to the number of base mismatches (Barlow et al., 2000). The HMA was carried out using amplified PCR product (see above). Products were quantified by comparison on a 1% agarose gel and the DNA concentration equalized by dilution. Equal amounts of two amplified products were mixed in a final volume of 10 μl. Mixtures of PCR products were denatured by heating at 94 C for 5 min, then rapidly cooled on ice for 10 min. Samples were then transferred to −20 C for a further 30 min. Heteroduplexes and homoduplexes were separated on 8% polyacrylamide gels (acrylamide:bisacrylamide 37.5:1) in a Protean-II gel apparatus (BioRad Laboratories, Hercules, California) at 100 volts for 2 hr in TAE buffer (40mM Tris, 2mM EDTA, Acetic Acid to pH 8). Bands were visualized by staining the gel in ethidium bromide (0.1 μg/ml) followed by UV illumination.

RESULTS

Phylogenetic analysis of the Turkish cohort was carried out on 16 nucleoprotein sequences from RABV from various locations in Turkey (Fig. 1, Table 1). Two further isolates from Samsun, held within the VLA archive since 1989, were included (Table 1). A 327 bp fragment from the 5′-end of the nucleoprotein coding region was used to compare the isolates using the Pasteur virus as an outgroup. Overall there was a maximum of 4.4% divergence between any two isolates, and between 8.2 and 11.1% divergence from the PV strain. A number of isolates had an identical sequence but were included in this analysis (Fig. 2). At this level there appears to be three lineages. One large group of viruses derived from areas in the west of Turkey including the major population centers of Istanbul, Bursa, and Izmir. Within this lineage isolates grouped together depending on the place of origin. The viruses RV202 and RV203, isolated in the Samsun region over a decade before the current cohort, aligned with the western group. A second group of viruses was derived from locations throughout the eastern regions of Turkey, including Gaziantep in the south and Ardahan in the north. Viruses were isolated from both dog and fox within this group. There is strong bootstrap support for this separation into two large groups. Finally, one isolate from the Ardahan region (RV1133) did not segregate with ei-

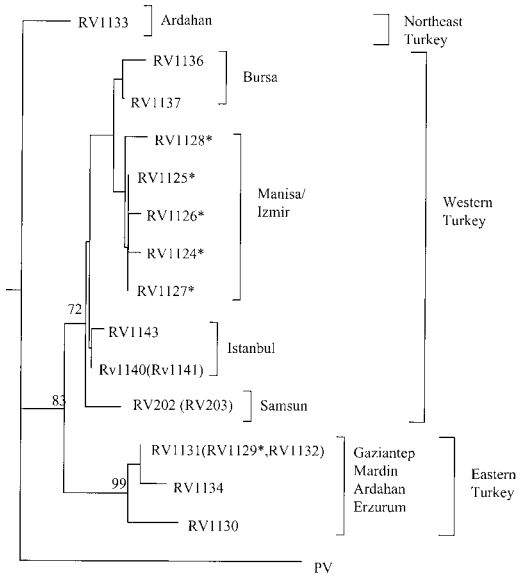

FIGURE 2. Phylogenetic relationships between 18 rabies virus isolates from Turkey based on 327 base pairs of the nucleoprotein coding sequence. The relationships are shown as a phylogram using the Pasteur virus (PV) sequence as an outgroup. Bootstrap values considered significant (>70) are shown. Isolates marked with an asterisk (*) were obtained from foxes.

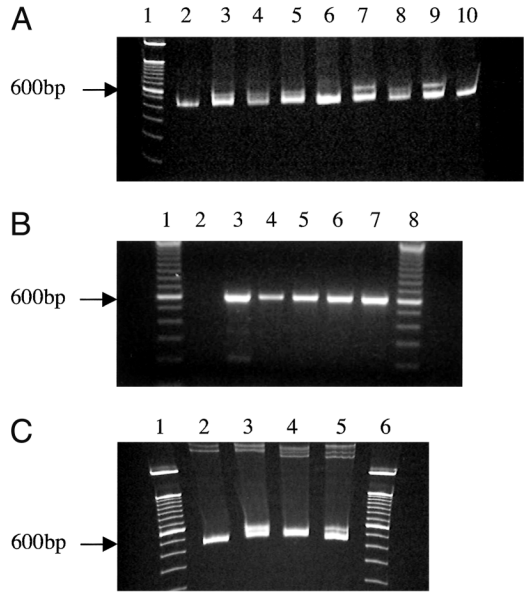

FIGURE 3. A. An 8% PAGE analysis of HMA samples of three isolates representing the groups identified by the phylogenetic analysis (Fig. 2). Lane 1 contains a 100 base pair ladder. Lanes 2–4 contain isolate RV1126 annealed to itself (lane 2), RV1130 (lane 3) and RV1133 (lane 4). Lanes 5–7 contain isolate RV1130 annealed to RV1126 (lane 5), itself (lane 6), and RV1133 (lane 7). Lanes 8–10 contain isolate RV1133 annealed to RV1126 (lane 8), RV1130 (lane 9), and itself (lane 10). B. A 1% agarose gel showing 100 base pair ladder (lanes 1 and 8), the PCR amplified product for the negative (lane 2) and positive (lane 3) controls, and for isolates RV1126 (lane 4), RV1130 (lane 5), RV1133 (lane 6), and RV1134 (lane 7). C. An 8% PAGE analysis of HMA samples presented in figure 3B. Lanes 1 and 6 contain 100 base pair ladder. Lane 2 contains isolate RV1134 annealed to itself, RV1126 (lane 3), RV1130 (lane 4), and RV1133 (lane 5).

ther of the two major groups including a second isolate from Ardahan.

Having established a phylogeny for the RABV cohort we then applied the HMA as an alternative method for grouping related RABV isolates. Figure 3A compares three isolates that are representative of the three lineages identified above. These are isolates RV1126 (west), RV1130 (east), and RV1133 (northeast). When annealed to themselves (lanes 2, 6, 10) the DNA fragments migrate as a single band. When annealed to a representative of another group there is clear retardation of heteroduplexes (lanes 3, 4, 5, 7, 8, 9). To confirm this, a fourth isolate (RV1134) was annealed to each of the three representative isolates. Figure 3B shows the initial PCR product for the three representative isolates and the test isolate (RV1134). Following annealing to itself the band migrates as a single band (Fig. 3C, lane 2). When annealed to the other three PCR products, multiple banding is seen with isolates RV1126 (lane 3) and RV1133 (lane 5), but a single band is observed when annealed to isolate RV1130 (lane 4). This demonstrates that isolate RV1134 is closely related to isolate RV1130, an observation confirmed by the phylogenetic analysis. Comparison of this finding with the partial sequences obtained for these isolates show that isolate RV1134 and isolate RV1130 have 97.9% sequence identity. Isolates RV1126 and RV1133 have a lower level of identity (95.7%) suggesting that this assay can differentiate sequences that are more

TABLE 2. Provisional grouping of rabies viruses from Europe and Russia (McElhinney et al., 2001).

European group	Region	Type isolate			GenBank accession number
		Reference number	Site isolated	Host	
1	Western Russia	RV234	Russia (Tula)	Dog	AY091628
2	Baltic/western Russia	RV437	Estonia	Raccoon dog (*Nyctereutes procyonoides*)	AY091627
3	Central and southern Russia	RV253	Russia (Tuva)	Wolf (*Canis lupus*)	AY091626
4	Central Europe	RV857	Czech Republic (Liberec)	Fox (*Vulpes vulpes*)	AY091629
5	Western Europe	RV312	Germany (Naven)	Fox	AY091630
6	Turkey	RV202	Turkey (Samsun)	Dog	AY091608

than 4% divergent. This provides the first reported evidence for the use of HMA in typing genetically related RABVs.

The differentiation of Turkish isolates into three lineages by both phylogenetic analysis and HMA raises a number of questions as to the origin of each lineage, especially the single isolate (RV1133) which does not appear to be related to the other two endemic groups. To address this, phylogenetic comparisons were made between the Turkish cohort and isolates derived from countries neighboring Turkey. This included isolates from the former Yugoslavia to the northwest, Georgia to the northeast, Iran to the east, and Israel to the south. The phylogenetic analysis of a 322 bp region of the N gene (Fig. 4) indicates that the eastern group is most closely related to isolates from Iran and to a lesser extent Israel. Conversely, the group of viruses from the west of Turkey does not seem to be related to any of the viruses sampled from external groups. The final group, containing the single isolate RV1133, grouped with two viruses isolated from Georgia. Figure 4 also compares the Turkish RABV cohort to RABV representing the six continental clades (McElhinney et al., 2001; Table 2) found over Europe and Russia (Fig. 4, inset). The western group of Turkish isolates cluster with the Europe 6 lineage that is specific to Turkey, whilst the eastern group does not cluster with the continental groups found in Europe and Russia. The isolate RV1133 also does not cluster with any of these lineages.

DISCUSSION

Molecular epidemiology is playing an increasing role in the understanding of the rabies virus and its relationship with host species. This approach has been applied at various geographic levels, from individual countries (Ito et al., 1999; David et al., 2000; de Mattos et al., 2000) to worldwide studies (Smith et al., 1992; Kissi et al., 1995). It has also provided information where a single host dominates the maintenance of rabies virus (Sacremento et al., 1992) or where more than one host is involved in transmission of the virus (von Teichman et al., 1995; Nadin-Davis et al., 2001). This situation has emerged in Turkey where rabies in domestic dogs predominated. Through concerted control measures including vaccination and eradication campaigns, the incidence of rabies in the dog population has been in steady decline. However, in recent years this trend has halted and a small, but significant increase in the incidence of dog rabies has occurred. This has also been associated with a rise in the number of rabies cases in foxes. The cause of these observations is unclear and may be a result of increased surveillance on wildlife reservoirs. Alternatively it may be caused by transmission from the principal host, the dog, to the fox population. A transmission

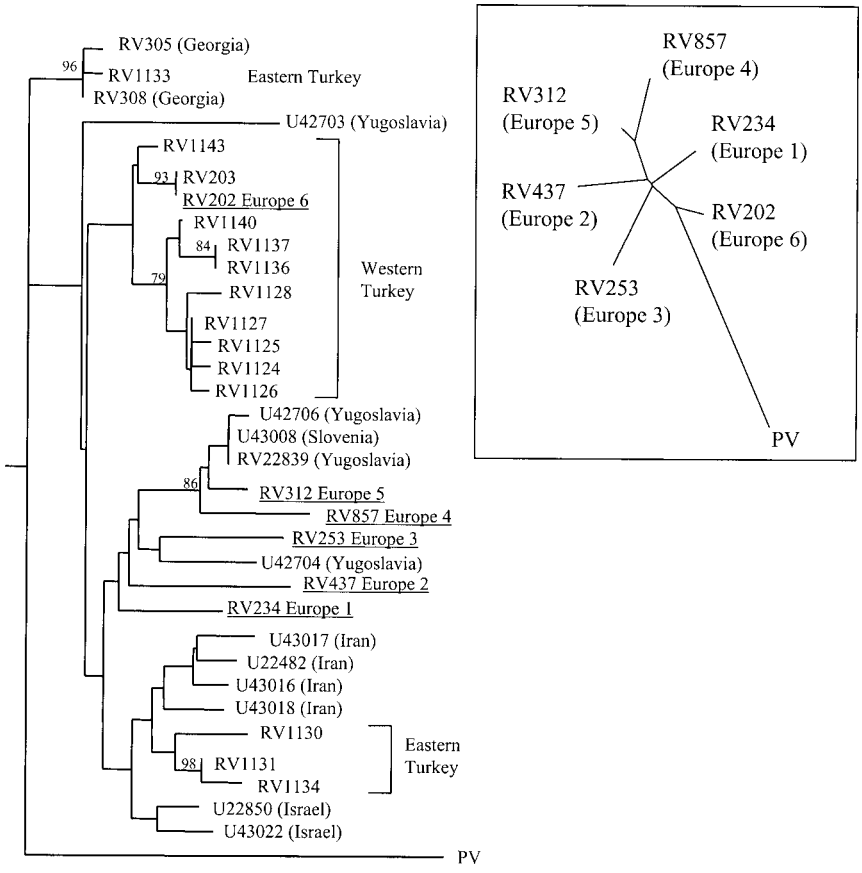

FIGURE 4. Phylogenetic relationships between the Turkish cohort and isolates derived from countries neighboring (Georgia, Iran) or in close proximity to Turkey (Israel, Yugoslavia, and Slovenia). RV isolates representing the six provisional groups covering Europe and Russia (inset) are also included in this analysis. The relationships are based on a 322 base pair sequence of the nucleoprotein-coding region. The relationships are shown as a phylogram using the Pasteur virus (PV) as an outgroup and bootstrap values >70 are shown. GenBank accession numbers are shown and were first published in Kissi et al. (1995).

event like this occurred in Central Europe in the late 1930s and led to the epizootic that spread throughout western Europe until the 1980s (Blancou, 1988). Phylogenetic analysis based on a 327 base pair sequence of the nucleoprotein coding region of the viral genome demonstrates that all the viruses investigated are genotype 1 viruses. Within Turkey, the RABV isolates form two large groups with one isolate that shows limited relatedness to these lineages. The first contains isolates from the west of the country from major population centers, including Izmir and Bursa, and stretching across the Bosporus Straits to Istanbul. This implies that the movement of animals by humans has clearly assisted the movement of rabies-infected animals because a body of water this size would be expected to block movement of the vector (Bourhy et al., 1999). Two RABV isolates from the Samsun area on the northern coast, which were isolated in 1989, cluster in this group indicating that this area is linked to the western region and that they share a common ancestor to this group. Whilst the dog remains the principal vector for transmission, the isolates from Izmir and Manisa were derived from foxes. The close relationship between isolates from these two hosts suggests that the transmission was a recent event. However,

it cannot infer the direction of transmission. It is presumed, although not conclusively proven, that the direction of transmission was from dog to fox. In South Africa, where two distinct lineages of RABVs co-exist, one in domestic dogs and one within wild species, particularly the yellow mongoose (*Cynictis penicillata*), such transmission events are commonly observed (von Teichmann et al., 1995).

The second group of Turkish RABVs was isolated from sites throughout the east of Turkey, again linked to urban centers. In one area, Erzurum (Turkey), identical isolates were recovered from both a dog and a fox, again suggesting recent transmission. A further discrepancy was the isolation of two poorly related viral isolates from the city of Ardahan. Both were derived from infected dogs, however one grouped more closely with isolates from Georgia, indicating that this may be a recent import from the north-east. This again suggests that human actions may be assisting the movement of virus into new areas.

The internal phylogeny of Turkish RABV isolates shows a separation into three lineages that was also observed by the HMA. This approach provides a less expensive and rapid alternative to sequencing every isolate. It does, however, require establishment of an initial phylogeny using limited sequencing of a defined region of the genome and identification of representative isolates to which subsequent viral sequences can be compared. With the spread of PCR as a technique to all countries throughout the world, the generation of an amplified DNA fragment from a viral isolate is a simple procedure. However, the inhibitory cost of sequencing to many surveillance laboratories prevents full phylogenetic analysis other than through collaboration with a limited number of laboratories, most located in the developed world. The HMA offers an alternative to identifying strains of rabies virus, is a rapid procedure, and could be used on large numbers of isolates in association with standard PCR techniques.

By comparison of the Turkish cohort of RABV isolates with those derived from countries surrounding Turkey, and to isolates representing nominal groups in Europe and the Russian Federation, it becomes clear that the eastern group is related to RABV found throughout the region. In contrast, the western group appears to be limited to Turkey. This suggests that the RABV isolates within this group have evolved for some time independently of viruses to the north and east. Surprisingly, the eastern group clusters more closely with isolates from the former Yugoslavia than the western group. This implies that there is some barrier preventing the movement of rabies out of the western area. What could have led to this dichotomy in phylogeny? Where rabies is limited to domestic dogs, surveillance will detect virus close to major human population centers. This also implies that movement of humans between population centers dominates movement of virus. Once rabies became established in the west of Turkey, further incursions may have been restricted by control of the limited border with western countries including Greece which is reportedly rabies free and Bulgaria for which data are limited. This contrasts with the east of Turkey, where there are considerable land borders with countries that are known to harbor large endemic populations of rabies, for example Georgia and Iran. Movement of populations between these countries, especially in time of conflict may have encouraged movement of virus across borders. In both groups, there is evidence that virus moved between hosts. This is probably not a new occurrence and such transmissions may have occurred regularly, but had not led to movement of virus throughout the country. Movement of this type has probably been restricted by the topography of Turkey, which is a country of over 777,000 km^2, much of it mountainous and above 1,000 m elevation. This could restrict

movement by vectors such as the fox. In summary, a combination of the type of host, the topography of the country, and movement of animals across land borders have shaped the current epidemiology of rabies in Turkey.

Recent study of rabies in Israel provides a possible model for what may occur in Turkey. A successful vaccination program introduced in 1956 eliminated the disease in domestic dogs (David et al., 2000). This was followed up by population reduction of the wild jackal with further reduction in the prevalence of rabies. Since the late 1970s the fox has been the principal reservoir of rabies in Israel. If this occurs in Turkey, future vaccination programs will need to include wild canid populations. However, the successful oral vaccination programs in Europe where the fox is the main reservoir and the topography mountainous, suggest that this may be an achievable goal.

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