Epidemiol. Infect. (2003), **131**, 777–790. © 2003 Cambridge University Press
DOI: 10.1017/S095026880300863X Printed in the United Kingdom

Molecular and antigenic characterization of rabies viruses from Iran identifies variants with distinct epidemiological origins

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(*Accepted 19 February 2003*)

SUMMARY

A molecular epidemiological study of 48 recent rabies isolates recovered from cases reported throughout Iran identified three distinct viral variants, the evolutionary origins of which were identified by phylogenetic comparison with rabies viruses originating from Europe and Asia. Members of group 1 (15 isolates) were recovered from the northern half of the country only, while those of group 2 (31 isolates) were widely dispersed; both groups clustered within the widely disseminated cosmopolitan lineage. The two isolates of group 3 were detected in the northeastern tip of the country only and belonged to the Arctic strain. Rapid variant discrimination tools, employing restriction fragment length polymorphisms applied to amplified fragments of the viral genome, were devised whilst antigenic characterization of representative viruses identified a small panel of monoclonal antibodies that were also discriminatory. The future application of such methods should provide valuable epidemiological information on rabies incidence in Iran.

INTRODUCTION

Rabies, known to Middle Eastern civilizations since ancient times, remains a significant problem throughout the region today. Even in countries such as Iran, where the Pasteur Institute in Tehran has provided human post-exposure treatment (PET) since 1923, human deaths from this disease continue to be reported [1]. This continuing problem is due to the role played by animal vectors in harbouring and transmitting the virus. In the northern forested areas bordering the Caspian Sea, wild canids including foxes and jackals are regarded as rabies vectors whilst in the mountainous western region wolves contribute to rabies dissemination. However, the majority of both human and domestic animal exposures are due to contact with rabid dogs and the dog population, which is often poorly controlled, constitutes a significant rabies reservoir [1].

Over the last 25 years there has been a substantial increase in human PETs in Iran. In 2000, 74 168 PETs were recorded throughout the country compared to 577 in 1973. By contrast, the numbers of laboratory-confirmed animal cases have remained relatively constant; for example, over the last decade an average of 354 cases per year were recorded with modest yearly variations. Similarly, human fatalities from the disease have remained fairly stable, averaging about 12 per year, throughout the 1990s (Fayaz, unpublished data). More cost-effective control measures would benefit from improved understanding of the

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vector species involved, the variants circulating in various regions of the country and the environmental and socio-economic factors contributing to their spread.

Rabies virus strain discrimination relies on both antigenic typing, employing panels of discriminatory monoclonal antibodies (mabs) directed primarily against nucleoprotein and glycoprotein components of the virus [2], and on genetic typing by comparing nucleotide sequences [3]. The rabies virus genome is a non-segmented negative sense RNA strand organized into five genes: N, P, M, G and L [4]. Studies on rabies virus variation have targeted several of these genes according to the purpose of the investigation (see [5]). The conserved N gene has proved useful for evaluating the worldwide diversity of this virus [6–9] and for phylogenetic studies on the entire Lyssavirus genus [10], within which all classical serotype 1 rabies viruses group as a single genotype. The greater variation of the G gene and the adjoining G-L intergenic region has been of value for highly detailed regional and local epidemiological studies [9, 11–14]. More recently, genetic characterization of the P gene of 128 lyssavirus isolates has identified a highly variable central region useful for sensitive molecular epidemiological analysis [15].

In this report, 48 rabies viruses, recovered from various species throughout Iran, were genetically characterized within the variable central portion of the P gene. Phylogenetic analysis of these data has examined the evolutionary relationships of the variants currently circulating in the country and their relationships to viruses present in other countries of the region. The genetic data also provided a means of designing a rapid method for variant discrimination. A complementary study on antigenic variation of these viruses identified discriminatory mabs useful for variant typing. Application of such rapid typing tools will more fully define the spatio-temporal distribution of these viral variants in Iran and provide information of value to rabies control strategies.

METHODS

Viral isolates

A summary of all isolates included in this study is presented in Table 1. Rabies was diagnosed in each case on brain tissue sections by the fluorescent antibody test (FAT) [16].

Antigenic characterization

Viruses from 10 selected brain specimens were isolated on mouse neuroblastoma (MNA) cells (Bio-Whittaker, Walkersville, MD) in 25 cm² filter capped tissue culture flasks (Falcon), using MEM with Earle's salts (Gibco BRL, Bethesda, MD), supplemented with 10% certified fetal bovine serum (Gibco BRL, US origin), 5% tryptose phosphate broth (Gibco BRL) and 1% antibiotic antimycotic solution (Sigma, Oakville, Ont). Infected cells were transferred to sterile 96 well tissue typing plates (Robbins Scientific, Sunnyvale, CA). Monolayers were fixed with 75% acetone after 1–2 days of incubation. After drying, 12 μl of each mab supernatant was dispensed into the appropriate well and plates were incubated for 50 min at 36·5 °C. Plates were quickly rinsed three times with buffer (50 mM Tris, 200 mM NaCl, 0·5% Tween), then washed three times for 10 min in buffer. Ten microlitres of buffer diluted, fluorescein-conjugated goat IgG fraction to mouse IgG (Cappel™, ICN Pharmaceuticals, Montreal Que) was added to each well and incubated for 40 min at 36·5 °C. Stained plates were rinsed and washed once for 10 min in buffer, then counterstained for 45 s with 0·5% Evans Blue stock solution in phosphate buffered saline diluted 1 in 200 in 50 mM Tris, 200 mM NaCL, 0·5% Tween buffer, pH 7·2–7·4.

Staining patterns were observed using an inverted UV microscope at 100 times magnification. A total of 373 anti-N, 67 anti-G, 53 anti-P, and 5 anti-M mab supernatants were used for the antigenic characterization.

RNA extraction

Total RNA was extracted from minced rabies virus-infected brain tissue (up to 0·1 g) using TRIzol™ according to the manufacturer's recommendations (Gibco BRL). Purified RNA was dissolved in diethyl-pyrocarbonate (DEPC)-treated sterile water and evaluated for concentration and purity spectrophotometrically.

RT–PCR

The RT–PCR protocol was performed essentially as described previously [17]. For P gene amplification the positive sense primer employed was Rab-Pfor1 5′-CTACTTCTCCGGGGAAACCAGAAG-3′ (corresponding to nt 1249–1272 of the PV reference strain, GenBank® accession no. NC 001542) though on

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Table 1. *Summary of rabies viruses employed in these studies*

Sample no.	City	Province	Date	Host species	Genetic grouping	Mab typing	GenBank® accession no.
V685	Khash	Sistan Baluchestan	Feb 2000	Goat	2	Iran-2	AY224155
V686	Ardebil	Ardebil	Feb 2000	Cow	1a	Iran-1	AY224140
V687	Masjid Soleiman	Khuzistan	Feb 2000	Donkey	2		AY224167
V688	Islamabad West	Kermanshah	Feb 2000	Wolf	1		AY224145
V689	Islamabad West	Kermanshah	Feb 2000	Cow	1	Iran-1	AY224142
V690	Islamabad West	Kermanshah	Feb 2000	Mule	1		AY224143
V691	Mobarakeh	Isfahan	Feb 2000	Jackal	2	Iran-2	AY224153
V692	Farsan	Chamahal Bakhtiari	Feb 2000	Fox	2		AY224162
V693	Shahroud	Semnan	Mar 2000	Dog	1		AY224150
V694	Divandareh	Kordestan	Mar 2000	Wolf	2a		AY224176
V695	Jieroft	Kerman	Mar 2000	Calf	2		AY224157
V696	Kahnouj	Kerman	Mar 2000	Camel	2		AY224159
V697	Gachsaran	Khuzistan	Apr 2000	Donkey	2		AY224163
V698	Shahroud	Semnan	Apr 2000	Cow	1		AY224148
V699	Masjid Soleiman	Khuzistan	Apr 2000	Cow	2	Iran-1v	AY224168
V700	Miyando-ab	Western Azarbayjan	Apr 2000	Dog	2a		AY224177
V701	Shirvan & Chardaval	Ilam	Apr 2000	Sheep	1		AY224144
V702	Jieroft	Kerman	Apr 2000	Calf	2		AY224160
V703	Shahroud	Semnan	Apr 2000	Sheep	1	Iran-1	AY224149
V704	Mashad	Khorasan	Apr 2000	Sheep	3	Iran-3	AY224183
V705	Masal	Gilan	May 2000	Cow	1a		AY224139
V706	Masal	Gilan	Apr 2000	Cow	1a		AY224141
V707	Shahroud	Semnan	Apr 2000	Dog	1		AY224151
V708	Mamassani	Fars	Apr 2000	Cow	2		AY224154
V710	Qazvin	Qazvin	Apr 2000	Cow	2b		AY224178
V711	Hashtroud	Eastern Azarbayjan	Apr 2000	Dog	2a	Iran-2	AY224173
V712	Fouman	Gilan	Apr 2000	Cow	1		AY224152
V713	Qazvin	Qazvin	Apr 2000	Cow	2b		AY224179
V714	Kahnouj	Kerman	Apr 2000	Cow	2		AY224158
V715	Estahban	Fars	Jun 2000	Jackal	2	Iran-1v	AY224164
V716	Shahroud	Semnan	Jun 2000	Calf	1		AY224146
V717	Mashad	Khorasan	May 2000	Mongoose	2a		AY224174
V718	Sanandaj	Kordestan	May 2000	Goat	2a		AY224171
V719	Delfan	Lorestan	May 2000	Dog	2		AY224170
V722	Shiraz	Fars	Mar 2000	Cow	2		AY224165
V723	Mashad	Khorasan	May 2000	Jackal	1		AY224147
V724	Mashad	Khorasan	May 2000	Dog	3		AY224184
V725	Iranshahr	Sistan Baluchestan	Jun 2000	Calf	2		AY224166
V726	Takab	Western Azarbayjan	Jun 2000	Dog	2a		AY224175
V727	Jieroft	Kerman	Jun 2000	Cow	2		AY224161
V728	Mashad	Khorasan	Jun 2000	Cow	2	Iran-2v	AY224181
V729	Iranshahr	Sistan Baluchestan	Jun 2000	Cow	2a		AY224172
V730	Sanandaj	Kordestan	Jun 2000	Sheep	2		AY224169
V732	Mashad	Khorasan	Jun 2000	Cow	2		AY224182
V734	Isfahan	Isfahan	Jun 2000	Donkey	2		AY224156
V735	Gachsaran	Kohkilouye & BouyerAhmad	1999	Human	2		AY224180
PIR5DG	Esfarayen	Khorasan	1993	Dog	2		AF369311
PIR14DG	Gorgan	Golestan/Gorgan	1993	Human	1a		AF369312

occasion amplification was achieved using an alternate primer Rab-Pfor2 5′-CATCARGCYGGNCCNAACTC(AT)TT-3′ (PV nt 1361–1383). The negative sense primer employed was Rab-Prev 5′-GGRAGCCAYAGGTCRTCGTCAT-3′ (PV nt 2575–2596). Thermal cycling was performed in a Perkin Elmer

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model 480 DNA thermocycler using the cycling parameters: 94 °C, 1 min, 45 °C, 1 min, 72 °C, 2 min (with a 5 s autoextension) for 30 cycles. In some cases sufficient PCR product was obtained only after a second round of amplification; internal sequencing primers, Rab-Pseqfor 5′-GAGATGGCAGAGGARACTGTAGATCT-3′ (PV nt 1568–1593) and Rab-Pseqrev 5′-GRGAAGGAGTGGATGTTGTCTCCTT-3′ (PV nt 1976–2000), were used in nested PCRs performed using a cycling profile as above except that an annealing temperature of 50 °C was employed. For amplification of the entire N gene the primers employed were Nseq0 5′-AACACCTCTACAATGGATGCCGAC-3′(positive sense, PV nt 59–82) and RabN5 5′-GGATTGACRAAGATCTTGCTCAT-3′ (negative sense, PV nt 1514–1536) for first round PCR and, as required, a second round PCR was undertaken by a hemi-nested approach using these first round primers and internal universal primers RabNfor 5′-TTGTRGAYCAATATGAGTACAA-3′ (PV nt 135–156) and RabNrev 5′-CCGGCTCAAACATTCTTCTTA-3′ (PV nt 876–896) to generate two amplicons overlapping in sequence within the central region of the N gene. PCR products were purified using a Wizard PCR (Promega Corp., Madison, WI) purification system prior to sequencing.

Nucleotide sequencing and phylogenetic analysis

Purified PCR products were sequenced either manually using a *fmol*® cycle sequencing system as directed by the manufacturer (Promega) with sequencing primers ^{32}P-labelled by treatment with [γ-^{32}P]ATP and T4 kinase or automatically with a model 4200S-2 system (Li-Cor, Lincoln, NE) employing custom IR Dye-labelled primers and a Thermo Sequenase Primer cycle sequencing kit (Amersham Biosciences). Nucleotide sequences were compiled into the DNASIS software package (Hitachi) which was used to predict encoded protein sequences and P gene restriction endonuclease cleavage patterns. Sequence alignments were performed using the CLUSTAL X programme [18] and phylogenetic analysis was undertaken using the PHYLIP 3.5 software package [19]. A neighbour joining analysis employing the Kimura 2 parameter and 1000 bootstrap replicates of the data was performed by sequential use of the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE. Distance values were reapplied to the consensus tree using FITCH before the tree was converted to a graphical output by TREEVIEW [20].

The sequences generated during this study have been deposited in GenBank®; accession numbers are provided in Table 1.

RESULTS

Phylogenetic analysis

The 48 Iranian rabies viruses employed in this study (see Table 1) were recovered from throughout the country except for the arid central region which has a sparse human population and which has a limited capacity to support wild canids. All isolates were amplified at the P locus and characterized by nucleotide sequencing over a 350 bp window corresponding to residues 1606–1955 of the reference PV strain. These data, together with the corresponding sequence of the reference PV strain as outgroup, were aligned using CLUSTAL X and then subjected to neighbour joining analysis using the PHYLIP 3.5 package as described in Methods. The resulting dendrogram is presented in Figure 1*a*; numbers above the branches indicate the times out of 1000 that each branch to the right was included in the consensus tree while the bar at bottom indicates the distance scale represented by the horizontal lines. The grouping designation, as referred to in the text below, is indicated at far right. The dendrogram divides all isolates into three main clades (1–3) each of which was supported with high bootstrap values ($\geqslant$91·3%). A radial representation of this tree (Fig. 1*b*) further illustrates the sub-division of these viruses into three main groups as well as identifying the main sub-groups described below. The geographical distribution of all these viral variants is illustrated in the map of Iran depicted in Figure 2.

Group 1 comprised 15 isolates, all of which were recovered from the northern and northwestern regions of the country. A sub-group of this clade (1a) was represented by four isolates all originating from areas neighbouring the Caspian Sea. Group 2 was constituted by 31 viruses recovered throughout the country and within this clade certain sub-groups were evident. Seven isolates formed sub-group 2a, five of which were reported as originating from a discrete area in the northwestern provinces whilst the other two isolates of this sub-group came from northeastern and southeastern parts of Iran. Only two isolates formed sub-group 2b, both coming from the city of Qazvin just south of the Caspian Sea region. Another set of four viruses appeared to cluster together (2c) as an outlying clade of group 2 but bootstrap values associated with this branch were very low (30·2%) and

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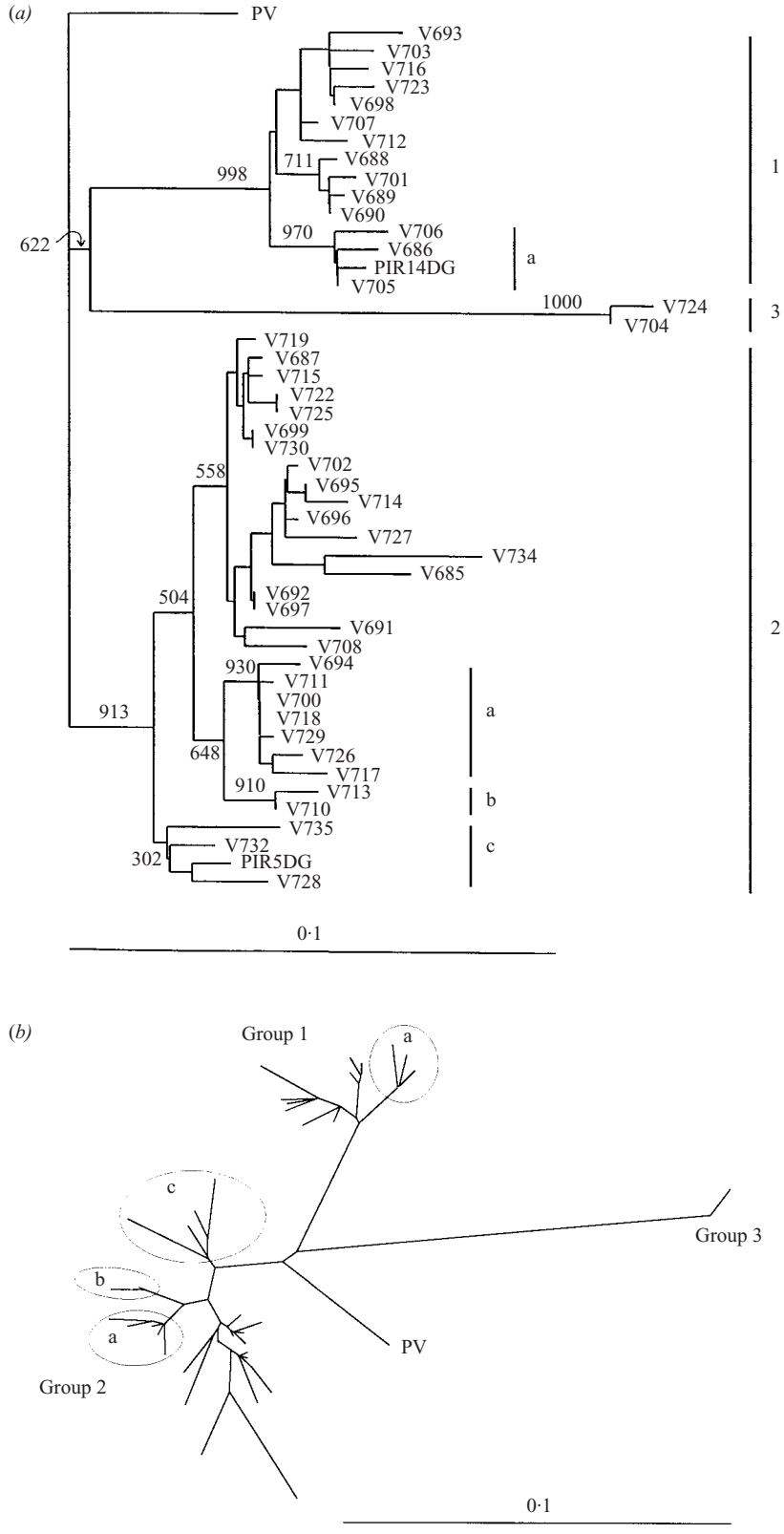

Fig. 1. Phylogenetic analysis of Iranian rabies viruses as depicted by (*a*) a dendrogram and (*b*) a radial tree.

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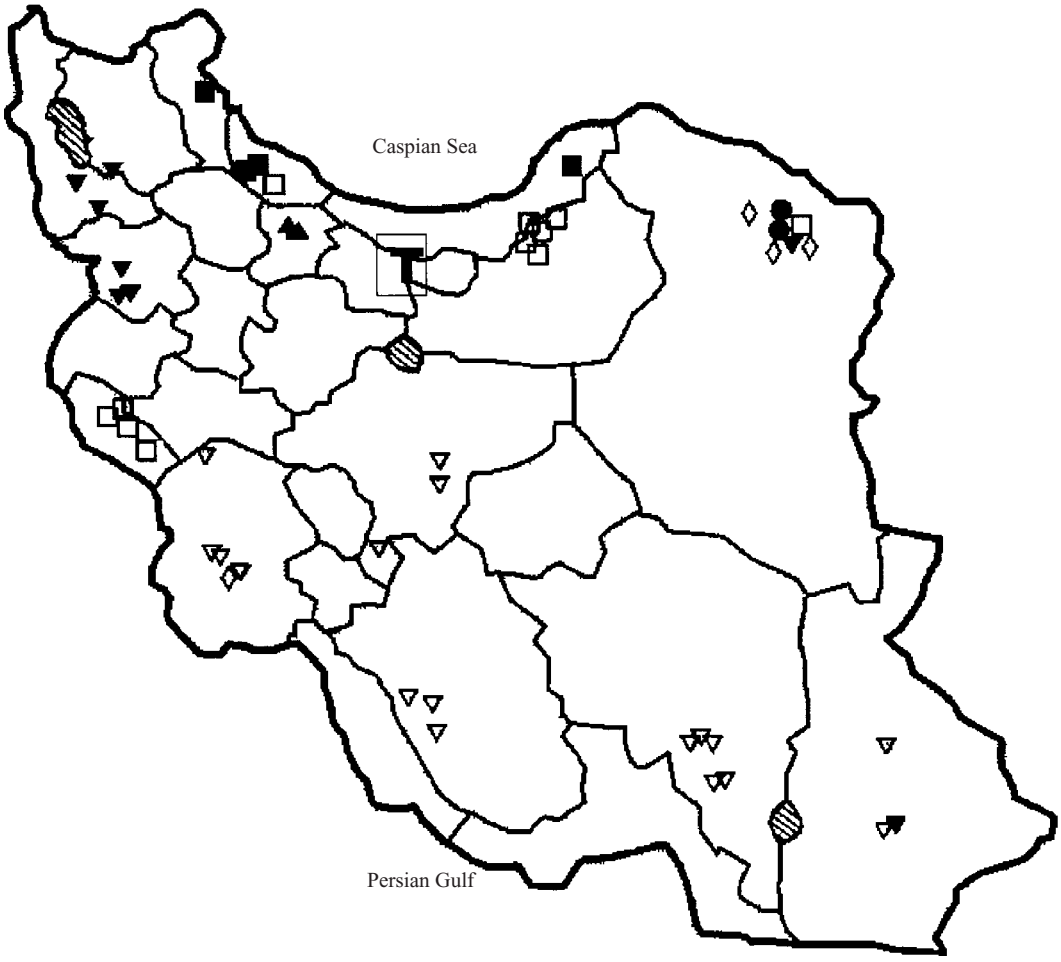

Fig. 2. Map of Iran showing the distribution of variants. The location from which all isolates of the study originated is shown according to their genetic grouping. Variants are depicted as follows: 1, □; 1a, ■; 2, ▽; 2a, ▼; 2b, ▲; 2c, ◇; 3, ●. The T enclosed in a box marks the position of the capital Tehran.

the isolates were distributed between two opposite sides of the country. Two closely-related isolates comprised group 3 and these were both recovered from the northeastern tip of Iran. The animal species infected by viral groups 1 and 2 are similar; in particular bovine cases make up a large proportion of cases (7 out of 15 for group 1 and 13 out of 31 for group 2 giving a total of 20 out of 48) whilst other domestic livestock species (sheep and equines) and various canid species (dog, jackal, wolf and fox) were also represented in each group. It is presumed, based on previous surveillance data, that dogs and other wild canids constitute the vector species in Iran while cases in livestock animals are a result of spillover from these reservoirs. The reporting of relatively high numbers of cases from livestock animals is probably due to their greater likelihood of investigation after death due to their economic and human health consequences. While it has been observed that bovines constitute a significant proportion of spillover cases of rabies in other jurisdictions, e.g. Ontario, Canada (unpublished data) it is unclear if this is due to a relatively high susceptibility of bovines to the disease. The small numbers of specimens in group 3 (one from a dog and one from a sheep) precludes comment on species distribution. Unfortunately due both to the limited numbers of canids included in the study and their infection with different rabies virus variants, it is impossible to conclude from these data whether all three rabies variants circulate in all canid reservoirs or whether certain variants are associated primarily with one specific vector species.

Molecular typing strategy for rapid group discrimination

The restriction endonuclease cleavage maps predicted from the partial P gene sequence for each isolate

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identified specific restriction sites which were selectively retained in some viral groups but not others. The possibility of using restriction fragment length polymorphisms (RFLPs) for rapid discrimination between viral groups, as has been previously described for mapping of sub-variants of the Arctic Fox strain in Ontario, Canada [14, 17], was thus investigated. Nested PCR products (450 bp), generated from representative isolates of each viral variant using Rab-Pseqfor/rev primers, were digested separately with five restriction endonucleases and then analysed by standard agarose gel electrophoresis. Figure 3 illustrates the restriction patterns generated for eight representative isolates using (*a*), *Bam*HI; (*b*), *Eco*O109I; (*c*), *Msp*I; (*d*), *Rsa*I; (*e*), *Sty*I. All group 1 viral PCR products were cleaved as follows: by *Bam*HI into two products (~225 bp), by *Msp*I into two products (~100, 350 bp), by *Rsa*I into two products (~200, 250 bp) and by *Sty*I into two products (~150, 300 bp); they were not cleaved by *Eco*O109I. Group 3 viruses exhibited similar cleavage patterns with *Bam*HI, *Eco*O109I and *Sty*I but differed in being uncleaved by *Msp*I and exhibiting a slightly different cleavage pattern upon treatment with *Rsa*I (two products of 190, 260 bp). PCR products of group 2 viruses, including those of sub-groups 2a and 2b exhibited a very different pattern; they were uncleaved by *Bam*HI, *Msp*I, *Rsa*I and *Sty*I but cut into three fragments (~75, 150, 225 bp) by *Eco*O109I. The PCR product generated from a group 2c virus generated an intermediate pattern in which it resembled group 3 after cutting by *Bam*HI, *Msp*I and *Sty*I but was uncut by *Rsa*I, as for other group 2 isolates, and it exhibited a distinctive cleavage pattern after treatment with *Eco*O109I (two bands of 75, 375 bp). It would thus appear that RFLP analysis can simply and rapidly discriminate between the three main rabies virus variants.

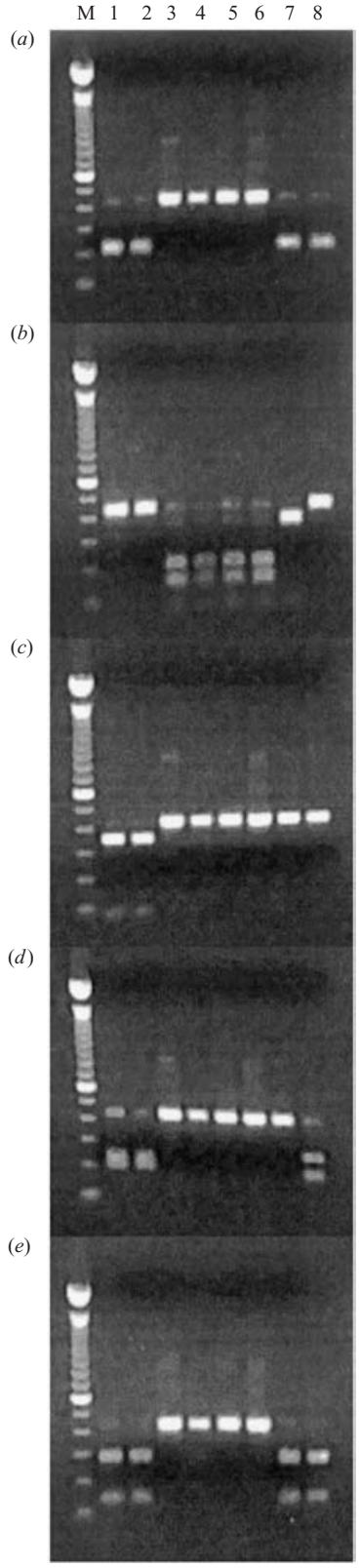

Fig. 3. RFLP-based discrimination of Iranian rabies virus variants. Lane 1, V712 (group 1); lane 2, V686 (group 1a); lane 3, V685 (group 2); lane 4, V722 (group 2); lane 5, V729 (group 2a); lane 6, V710 (group 2b); lane 7, V728 (group 2c); lane 8, V704 (group 3). Lane M, 100 bp DNA marker.

P protein variation

From the partial P gene sequences generated by these studies the primary sequence of a portion of the encoded phosphoprotein product (117 amino acids corresponding to residues 31–147 of the P protein of the PV strain) was predicted for each isolate. All 48 encoded partial P protein sequences, together with corresponding sequence of the phosphoprotein of the reference PV strain (GenBank® accession no. NC 001542) were aligned using CLUSTAL X and analysed by the PROTPARS program of PHYLIP 3.5.

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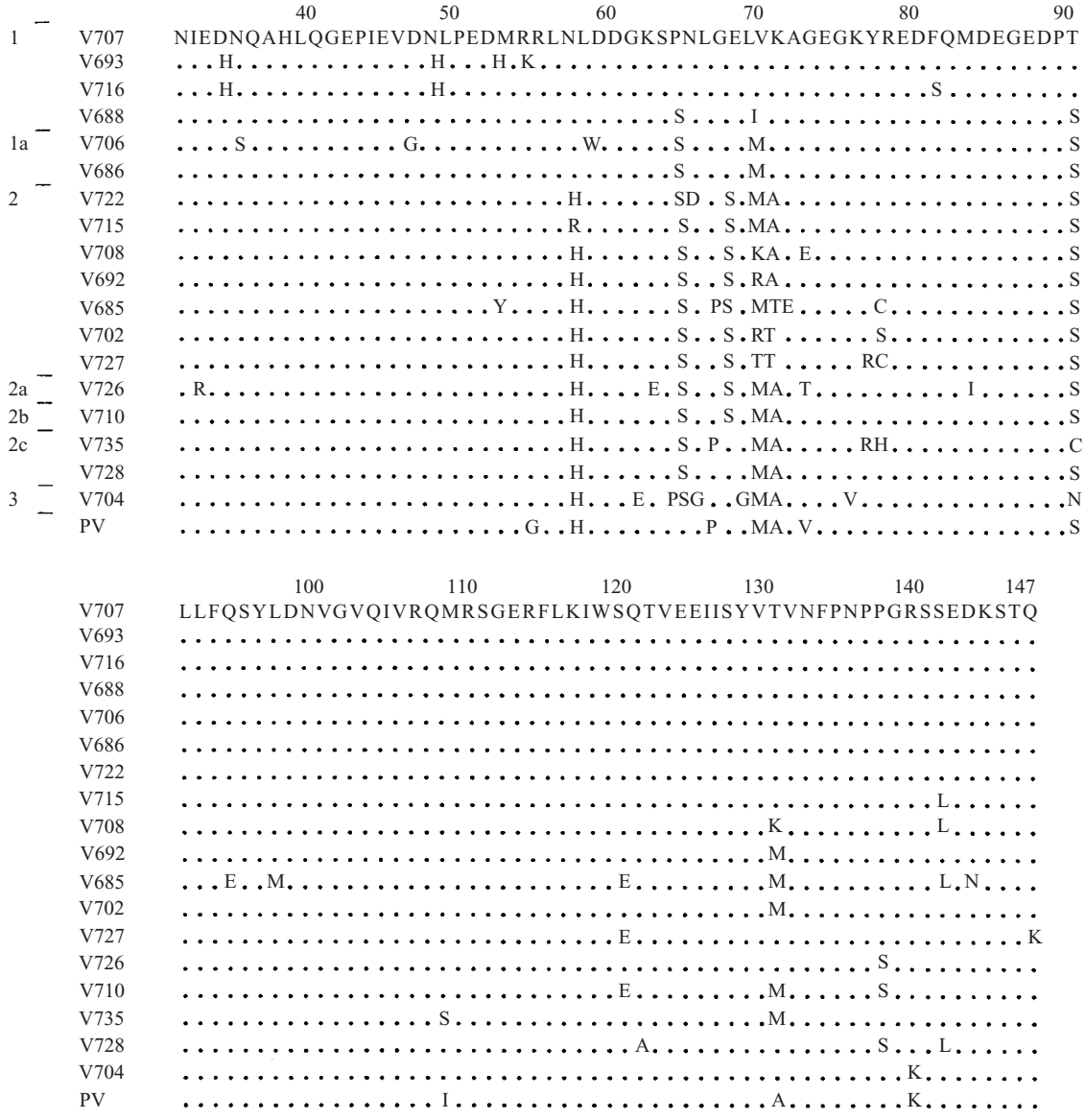

Fig. 4. Comparison of the predicted P protein sequences of representative Iranian rabies viruses.

The alignment presented in Figure 4 shows the sequence of isolate V707 (group 1) as the reference and deviations from this sequence for 17 other representative isolates and the PV strain. The residue numbering refers to that of the complete P protein (previously referred to as M1) as determined for PV. Despite the targeting of a relatively divergent portion of the P gene, the encoded product exhibits relatively modest overall variation with most amino acid substitutions occurring between residues 57 and 77. Consistent with their relatively distant relationship to the other specimens, group 3 viruses exhibit the most distinctive P product with several substitutions (residues 61–70, 75 and 90) restricted to these viruses only. Certain substitutions appear to respect the phylogenetic groupings of some isolates, e.g. T in place of A at residue 72 in all group 2a viruses, N in place of H or R at residue 57 for all group 1 viruses. Many other substitutions appear more randomly in specific isolates only.

Global context of Iranian rabies viruses

The extent of variation observed between these Iranian rabies viruses was placed in a global context by comparing selected representatives of each of these viral groupings with other viruses recovered from around the world and in particular from the Middle East, Europe and Asia. Partial P gene sequences for selected isolates, as described previously (see [15]),

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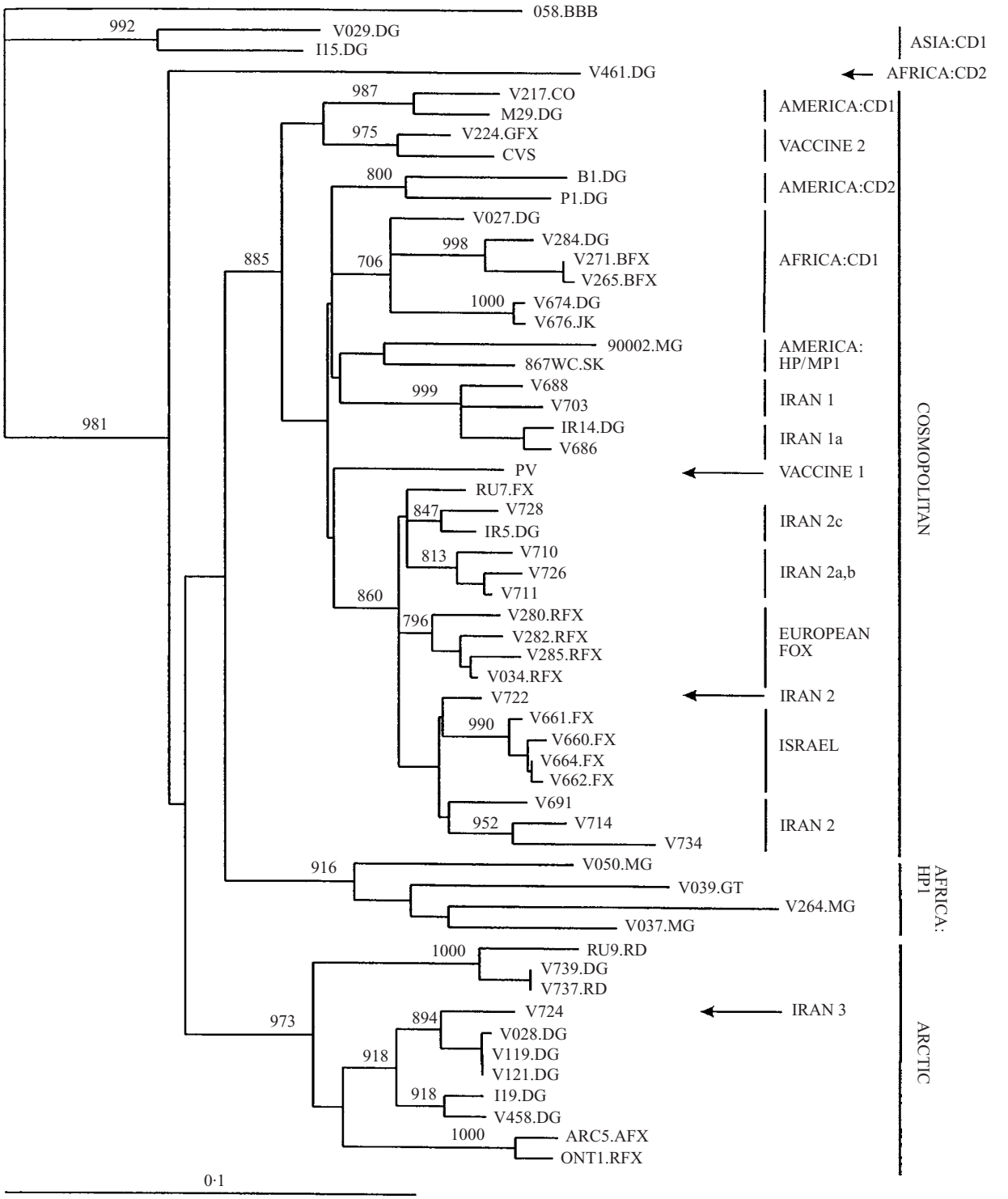

Fig. 5. Phylogenetic placement of Iranian rabies virus variants within a global context. Bootstrap values >700 are shown for all branches; strain/grouping names, as described in the text [and ref. 15], are shown at far right. The *suffixes* attached to each specimen name indicate the species of origin thus: AFX, arctic fox; BFX, bat-eared fox; CO, coyote; DG, dog; FX, fox of undefined species; GFX, grey fox; GT, genet; JK, jackal; MG, mongoose; RD, raccoon dog; RFX, red fox; SK, skunk. Two Korean isolates (V737.RD and V739.DG) not described previously were also included. An isolate (058.BBB) from an insectivorous bat of the Americas was employed as an outgroup.

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Table 2. *Summary of the reactivities exhibited by a selection of discriminatory anti-rabies mabs with representatives of the genetic variants of the Iranian rabies viruses*

	Antigen used for mab generation	Isolate no.									
		V686	V703	V689	V715	V699	V691	V711	V685	V728	V704
N-mabs											
5DF12	SAD* RNP	+++	+++	+++	+++	+++	+++	+++	+++	−	+++
26AD4	Dog, Sri Lanka, RNP	+++	+++	+++	+++	+++	−	−	−	−	+++
26BD6	Dog, Sri Lanka, RNP	+++	+++	+++	+++	+++	+++	+++	++	+++	−
26BG8	Dog, Sri Lanka, RNP	+++	+++	+++	+++	+++	−	−	−	−	++
26EB10	Dog, Sri Lanka, RNP	+++	+++	+++	+++	+++	−	−	−	−	+++
26ED5	Dog, Sri Lanka, RNP	+++	+++	+++	+++	+++	−	−	−	−	+++
M835	Duvenhage, RNP	+++	+++	+++	++	+++	−	−	−	−	+++
M868	Duvenhage, RNP	+++	+++	+++	+++	+++	−	−	−	−	+++
M872	Duvenhage, RNP	+++	+++	+++	+++	+++	−	−	−	−	+++
M873	Duvenhage, RNP	+++	+++	+++	+++	+++	−	(+)	−	−	+++
M878	Duvenhage, RNP	−	−	−	+++	+++	+++	+++	+++	+++	+++
M1396	Mongoose, Africa, RNP	+++	+++	++	++	++	−	−	−	−	(+)
M1494	EBLV-2†, RNP	++	++	+	+	−	−	−	−	−	(+)
M1586	SHB‡, RNP	+++	+++	+++	+++	+++	−	−	−	−	−
M1594	SHB, RNP	+++	+++	+++	+++	+++	−	−	−	−	−
M1596	SHB, RNP	(+)	−	−	−	−	−	−	−	−	−
P-mabs											
M960	Canarctic P-protein	−	−	−	−	−	−	−	−	−	+++
M977	Canarctic P-protein	−	−	−	−	−	−	−	−	−	+++
	Antigenic group	Iran-1	Iran-1	Iran-1	Iran-1v	Iran-1v	Iran-2	Iran-2	Iran-2	Iran-2v	Iran-3

* SAD, Street Alabama Dufferin strain; † EBLV-2, European Bat lyssavirus type 2b rabies-related virus; ‡ SHB, silver-haired bat strain. Reactivities were scored as negative or positive with weak + to strong +++ fluorescent staining.

together with representative specimens from each of the Iranian groupings were analysed by NJ analysis as detailed in Methods. Using nucleotide sequence data over a 365 bp segment corresponding closely to that used above, a phylogenetic analysis was performed on a total of 56 rabies viruses (Fig. 5). Many of the additional isolates utilized in this tree have been described previously [15] and several represent sub-groups of the cosmopolitan lineage believed to have arisen initially in Europe in the 17th century before being widely disseminated to Asia, Africa and the Americas by rabies-infected companion animals during the colonial period [6, 21]. Whilst the Iranian viruses of groups 1 and 2 cluster within the cosmopolitan lineage with good bootstrap support (88·5%), group 1/1a viruses form a rather distinct clade within this lineage. All variants of group 2 viruses associate far more closely with recent European and Israeli fox isolates (bootstrap value of 86·0%). In contrast the group 3 specimen (V724) is contained within a group of viruses known as the Arctic lineage that circulates in wild canids and dogs in Northern

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Table 3. *Distance values computed for N gene and N protein sequences of seven Iranian rabies virus variants*

		Pair wise distance values for the N gene						
		NV686	NV703	NV699	NV715	NV685	NV711	NV728
Pair wise distance values for the N protein	NV686	0	0·0206	0·0682	0·0612	0·0681	0·0667	0·0651
	NV703	0·0021	0	0·0643	0·0574	0·065	0·0628	0·0598
	NV699	0·01504	0·01285	0	0·0156	0·0344	0·025	0·0546
	NV715	0·0064	0·0043	0·01287	0	0·03	0·0192	0·0478
	NV685	0·0086	0·0064	0·01503	0·0064	0	0·0249	0·056
	NV711	0·01074	0·0086	0·0172	0·0086	0·0021	0	0·0546
	NV728	0·01078	0·0086	0·01725	0·0086	0·0065	0·0086	0

Distances were determined using the DNADIST and PROTDIST programs of PHYLIP 3.5 and compiled into a single table using Excel software.

temperate, sub-arctic and arctic zones of Russia, Canada, Northern India, Korea and Nepal. The Iranian specimen is most closely related to isolates from dogs in Nepal and Northern India (bootstrap value of 89·4%).

Antigenic analysis

Since routine rabies virus typing is frequently achieved by antigenic analysis, the utility of an extensive collection of anti-rabies mabs for typing of these Iranian viruses was investigated. Ten viral isolates representative of all genetic variants were propagated in cell culture and then assayed for reactivity as described (see Methods). Of the 498 mabs studied, only 16 anti-N and 2 anti-P mabs proved to be useful for differentiating between these isolates and their reactivity patterns are summarized in Table 2. Three main groups of antigenic variants were identified and labelled Iran-1,2,3 with minor variations in reactivities, designated Iran-1v and -2v, being noted for certain isolates. Iran 1 and 3 groups were generally reactive to mabs derived using RNP antigen from the Sri Lankan dog and Duvenhage viruses with the exception of mab 26BD6 which did not react with the Iran-3 group. However, the Iran-1 viruses differed from those of Iran-3 by also reacting to most of the mabs generated using RNP of a North American silver-haired bat rabies virus strain. The most striking difference between Iran-1 and 3 groups however was the reactivity by Iran-3 virus only with two anti-P mabs (M960, M977) generated using the Canadian arctic fox strain. A sub-group of viruses (Iran-1v) differed from the main group (Iran-1) primarily by their reactivity to M878. In contrast Iran-2 variants reacted to only a few of these Mabs (26BD6, M878 and 5DF12 except for V728).

To investigate the genetic basis for these antigenic distinctions, a total of seven selected specimens, representing Iran-1 (V686, V703), Iran-1v (V699, V715), Iran-2 (V685, V711) and Iran-2v (V728) antigenic groups were characterized by sequencing of the N locus. N gene PCR products for all seven isolates, generated as described, were sequenced over the complete nucleoprotein coding region and distance values were computed in a pair wise analysis for all samples both for nucleotide and predicted protein sequences as shown in Table 3. The N gene distance values presented are in accord with the phylogenetic data of the P locus by their support of a closer relationship between Iran-1v and Iran-2 isolates rather then between groups Iran-1/1v. However, the very low N protein distance values do not exhibit such a clear association. An alignment of these protein sequences, performed by CLUSTAL X and illustrated with the use of the PROTPARS program of PHYLIP 3.5, shows the predicted amino acid substitutions between all seven isolates (see Fig. 6). Dots represent identity with the reference sequence (isolate V686) at each position. The antigenic group of each isolate is shown at bottom right. Whilst several substitutions apparently exhibit no group association, the following are notable: at residue 101 Iran-1/1v = D, Iran-2/2v = N; at residue 398 Iran 1/1v/2v = E, Iran-2 = G; at residue 448 Iran-1 = S whilst all other viral groups have N at this position.

DISCUSSION

The molecular epidemiological findings of this study suggest the current circulation within Iran of at least three distinct rabies virus variants, two of which associate with the cosmopolitan lineage. The group 2

https://doi.org/10.1017/S095026880300863X Published online by Cambridge University Press

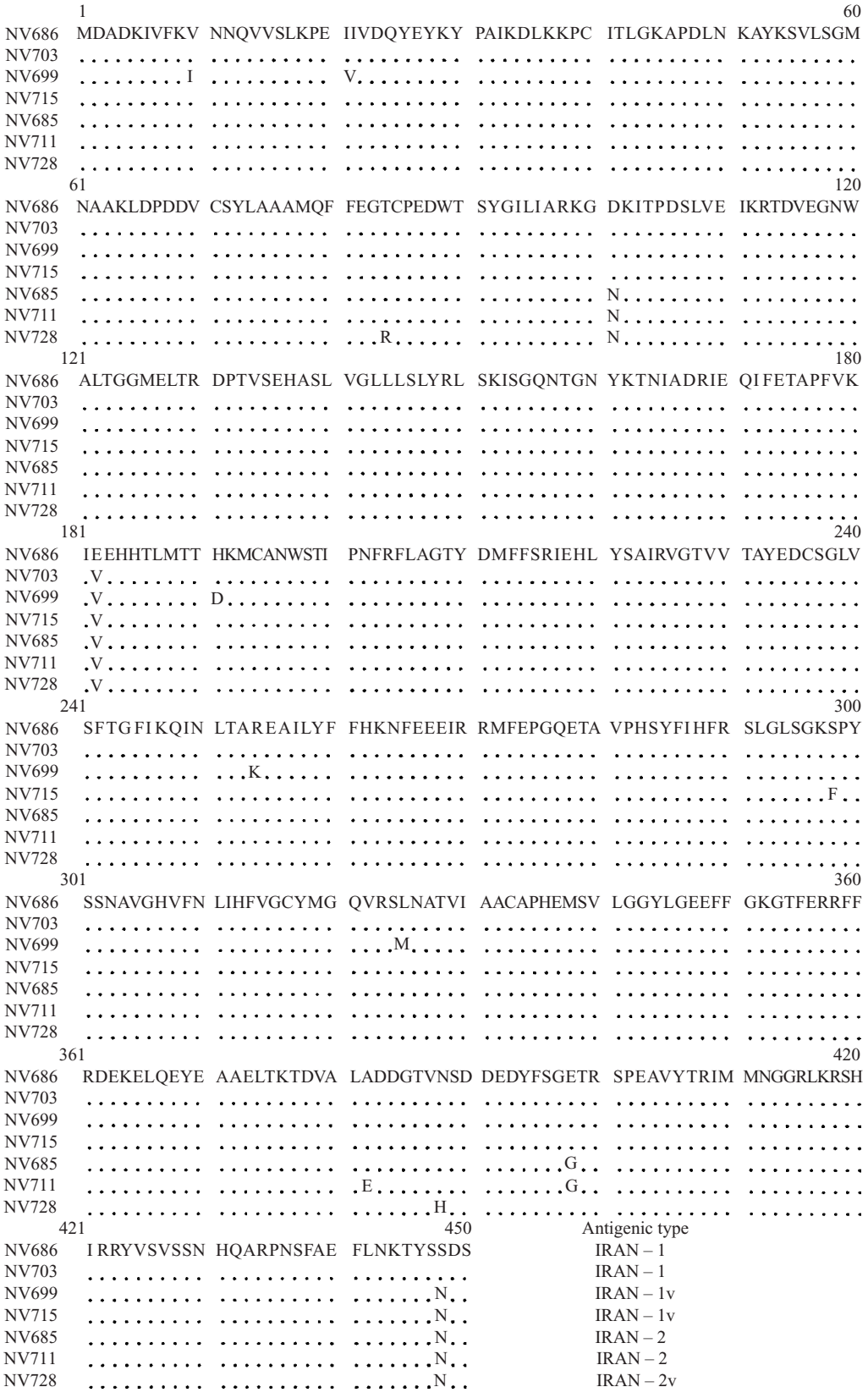

Fig. 6. Comparison of the predicted nucleoprotein sequences of seven representatives of the main antigenic groups of Iranian rabies viruses.

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viruses are widely disseminated throughout the country and this was the only variant recovered in central and southerly regions. This viral group clearly shares close evolutionary origins with rabies viruses recovered from wild carnivores in Europe and the Middle East but it was notable that sub-variants of this group segregated to multiple distinct branches of the cosmopolitan clade (Fig. 5) thereby suggesting that these variants have evolved from temporally or spatially independent movements of this rabies lineage into Iran. Group 1 viruses, which were found near western and northern border areas of the country, were clearly more distantly related to the European fox/Middle Eastern strain and formed a distinct cluster quite separate from group 2 Iranian viruses. Indeed, the group 1 specimens from Iran were as divergent from the European/Middle Eastern clade as were rabies viruses recovered from African canids (group Africa:CD1) and herpestid hosts of the Americas (clade America:HP/MP1). The viruses represented by Iranian groups 1 and 2 thus probably emerged from multiple independent incursions of the cosmopolitan lineage into the country. These observations are consistent with the results of a molecular epidemiological study of Israeli rabies viruses in which the phylogenetic proximity of European and Middle Eastern strains was noted [22]; of the two Iranian specimens included in that study one segregated quite closely with the Israeli variants and the other was genetically more distant. Those two isolates may be representative of the two major rabies virus groups described in this report. Unfortunately the fairly low numbers of wild canids included in this study make it difficult to draw any conclusions regarding their role as reservoirs for each of these rabies virus groups. Viruses of group 1 were identified in 1 wolf, 2 dogs and 1 jackal whilst viruses of group 2 were found in 1 wolf, 5 dogs, 1 fox and 2 jackals. The application of the rapid typing tools described in this paper will assist in future studies on larger numbers of rabid canids to determine whether specific viral groups persist in particular host species.

Only two specimens of a third group were recovered in a highly restricted area in the northeastern tip of the country and these viruses are phylogenetically very distinct from all other Iranian specimens. They are closely related to members of the Arctic lineage and in particular to the viruses recovered from dogs in Nepal although this strain circulates in many countries of the region. The limited distribution of this group would suggest that it is a recent incursion from neighbouring countries to the east but further monitoring of this area and eastern areas to the south will be important to determine the full geographical range of this variant and its temporal spread.

Antigenic characterization of representative Iranian isolates identified 18 mabs useful for group discrimination and three principal antigenic groups were identified. Interestingly, the two antigenic variants Iran-1 and Iran-1v share epitopes that are missing on Iran-2 and Iran-2v. Whilst Iran-1 and Iran-2/2v antigenic variants corresponded to genetic groups 1 and 2 respectively, curiously the two isolates yielding the Iran-1v pattern were predicted by phylogenetic analysis to be more closely related to group 2 viruses by clustering to a particular sub-branch within the group 2 clade. Interestingly, only a single anti-N Mab, M878, recognized the Iran-1v and all Iran-2/2v isolates but not Iran-1 viruses. The N protein epitope defined by this mab is likely to be influenced, directly or indirectly, by residue 448 which is the only position in the nucleoprotein that exhibits a particular substitution restricted to Iran-1 isolates. Based on the very limited degree of amino acid substitution observed between the N proteins of all isolates the discriminatory anti-N mabs of this panel must be recognizing only a small number of epitopes. Indeed, the only substitution consistent with the Iran-1/1v and Iran-2/2v distinction is that observed at residue 101, an observation suggestive that these discriminatory mabs must be recognizing epitope(s) influenced by this residue. Notably neither residue 101 or 448 have been associated with antigenic sites of the N protein previously. Where one or very few epitopes are targeted by an antigenic typing scheme, a single nonsynonymous nucleotide change can substantially alter an antigenic type. In such a situation group determinations are more reliable predicted by genetic studies that compare a length of sequence of several hundred bases. Discrepancies in viral typing schemes between genetic and antigenic techniques are uncommon; a previous characterization of Canadian bat rabies viruses by these two methods gave generally very consistent findings [23].

Specimen V704 was the only isolate examined that is recognized by M960 and M977, two Mabs that are specific for antigenic site II on the P-protein [24]. This epitope was so far recognized only on Canadian viruses circulating in the Arctic and in fox populations of Ontario and Quebec, as well as on isolates from Korea, and this observation clearly substantiates the phylogenetic placement of this group in the Arctic group. The localization of this group to the

northwestern tip of the country suggests incursion from Iran's eastern neighbours. Indeed, these studies have shown the incursion from neighbouring countries into Iran of phylogenetically distinct rabies virus strains originating from either the west (cosmopolitan group) or the northeast (arctic group) and emphasize the need to monitor future incursions into the country as well as more precisely define the geographical and temporal movements of those variants already circulating in Iran. Early recognition of new epizootics is essential to direct mobilization of the appropriate control strategies to those areas in most immediate need. In addition it would be of interest to further examine whether the various rabies virus groups identified in this study tend to be associated with specific reservoir species and the rapid typing tools developed in this report could be applied to such studies as well as investigations of rabies in other countries of the region that harbour some or all of the variants described here. Information derived from such studies would greatly improve our understanding of the epidemiology of this disease in countries bordering the middle-East and western Asia.

REFERENCES

1. Fayaz A. La rage en Iran. In: Kuwert E, Mérieux C, Koprowski H, Bögel K, eds. Rabies in the Tropics. Berlin: Springer-Verlag, 1985: 583–586.
2. Rupprecht CE, Dietzschold B, Wunner WH, Koprowski H. Antigenic relationships of lyssaviruses. In: Baer GM, ed. The natural history of rabies, 2nd edn. Boca Raton: CRC Press, 1991: 69–100.
3. Sacramento D, Bourhy H, Tordo N. PCR technique as an alternative method for diagnosis and molecular epidemiology of rabies virus. Mol Cell Probes 1991; **5**: 229–240.
4. Tordo N. Characteristics and molecular biology of the rabies virus. In: Meslin FX, Kaplan MM, Koprowski H, eds. Laboratory techniques in rabies, 4th edn. Geneva: WHO, 1996: 28–51.
5. Nadin-Davis SA. Rabies and rabies-related viruses. In: Thompson RCA, ed. Molecular epidemiology of infectious diseases. London: Arnold Press, 2000: 245–257.
6. Smith JS, Orciari LA, Yager PA, Seidel HD, Warner CK. Epidemiologic and historical relationships among 87 rabies virus isolates as determined by limited sequence analysis. J Infect Dis 1992; **166**: 296–307.
7. Smith JS, Orciari LA, Yager PA. Molecular epidemiology of rabies in the United States. Semin Virol 1995; **6**: 387–400.
8. Kissi B, Tordo N, Bourhy H. Genetic polymorphism in the rabies virus nucleoprotein gene. Virology 1995; **209**: 526–537.
9. Bourhy H, Kissi B, Audry L, et al. Ecology and evolution of rabies virus in Europe. J Gen Virol 1999; **80**: 2545–2557.
10. Bourhy H, Kissi B, Tordo N. Molecular diversity of the *Lyssavirus* genus. Virology 1993; **194**: 70–81.
11. Sacramento D, Badrane H, Bourhy H, Tordo N. Molecular epidemiology of rabies virus in France: comparison with vaccine strains. J Gen Virol 1992; **73**: 1149–1158.
12. Badrane H, Tordo N. Host-switching in Lyssavirus history from the chiroptera to the carnivora orders. J Virol 2001; **75**: 8096–8104.
13. Von Teichman BF, Thomson GR, Meredith CD, Nel LH. Molecular epidemiology of rabies virus in South Africa: evidence for two distinct virus groups. J Gen Virol 1995; **76**: 73–82.
14. Nadin-Davis SA, Sampath MI, Casey GA, Tinline RR, Wandeler AI. Phylogeographic patterns exhibited by Ontario rabies virus variants. Epidemiol Infect 1999; **123**: 325–336.
15. Nadin-Davis SA, Abdel-Malik M, Armstrong J, Wandeler AI. Lyssavirus P gene characterisation provides insights into the phylogeny of the genus and identifies structural similarities and diversity within the encoded phosphoprotein. Virology 2002; **298**: 286–305.
16. Dean DJ, Ableseth MK, Atanasiu P. The fluorescent antibody test. In: Meslin FX, Kaplan MM, Koprowski H, eds. Laboratory techniques in rabies, 4th edn. Geneva: WHO, 1996: 88–95.
17. Nadin-Davis SA. Polymerase chain reaction protocols for rabies virus discrimination. J Virol Methods 1998; **75**: 1–8.
18. Jeanmougin F, Thompson JD, Gouy M, Higgins DG, Gibson TJ. Multiple sequence alignment with Clustal X. Trends Biochem Sci 1998; **23**: 403–405.
19. Felsenstein J. PHYLIP: Phylogeny inference package, Version 3.52c, University of Washington, Seattle, 1993.
20. Page RDM. TREEVIEW: an application to display phylogenetic trees on personal computers. Comp Appl Biosci 1996; **12**: 357–358.
21. Smith JS, Seidel HD. Rabies: a new look at an old disease. In: Melnick JL, ed. Progress in medical virology. Basel: Karger, 1993: **40**: 82–106.
22. David D, Yakobson B, Smith JS, Stram Y. Molecular epidemiology of rabies virus isolates from Israel and other middle- and near-eastern countries. J Clin Microbiol 2000; **38**: 755–762.
23. Nadin-Davis SA, Huang W, Armstrong J, et al. Antigenic and genetic divergence of rabies viruses from bat species indigenous to Canada. Virus Res 2001; **74**: 139–156.
24. Nadin-Davis SA, Sheen M, Abdel-Malik M, Elmgren L, Armstrong J, Wandeler AI. A panel of monoclonal antibodies targeting the rabies virus phosphoprotein identifies a highly variable epitope of value for sensitive strain discrimination. J Clin Microbiol 2000; **38**: 1397–1403.