

A new outbreak of rabies in rare Ethiopian wolves (*Canis simensis*)

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Abstract Between October 2008 and May 2009, five brain samples from the carcasses of the rare Ethiopian wolf (*Canis simenensis*) were submitted for rabies virus testing. Rabies virus was detected in all five samples, and this confirmed that a further outbreak of rabies had occurred within the wolf population in the Bale Mountains of Ethiopia. Sequence comparison of a partial fragment of the nucleoprotein-coding gene demonstrated that all viruses showed 100% sequence identity, suggesting a single introduction of rabies virus.

Rabies is endemic within the domestic dog population of Ethiopia and continues to be a public-health threat and an economic burden [15]. The endangered Ethiopian wolf (*Canis simensis*) is the world's rarest canid, with an

estimated population of fewer than 500 individuals in seven small isolated populations, all within Ethiopia [1, 8, 13]. During 1991/2 and again in 2003/4, rabies outbreaks were responsible for the death or disappearance of approximately 75% of known wolves in the closely monitored packs of the Web Valley, in the Bale Mountains National Park, home to more than half of the global Ethiopian wolf population [10, 12]. Rabies was confirmed in the first outbreak by the use of an immunofluorescence test [12] and by molecular detection of virus in a decomposed wolf carcass [17]. Throughout Africa, domestic dogs act as the principal reservoir for this virus [14], and evidence suggests that this holds for areas where Ethiopian wolves persist [6, 7, 9]. Phylogenetic analysis of rabies viruses detected in 13 of 15 brain samples obtained from wolf carcasses during 2003/4 demonstrated that the outbreak had resulted from a spillover of rabies viruses endemic in domestic dogs in Ethiopia [10]. This outbreak was controlled with a parenteral vaccination campaign organized by the Ethiopian Wolf Conservation Programme (EWCP, <http://www.ethiopianwolf.org>) [2, 5, 10].

In October 2008, four brain samples obtained from wolf carcasses from the Web Valley in the Bale Mountains were submitted to the Veterinary Laboratories Agency for confirmation of a diagnosis of rabies. All four were rabies positive by nested RT-PCR using methods described previously [3], and a virus nucleoprotein-coding sequence was generated for each sample. Two further brain samples from a single carcass found in West Morebawa (Approx. 22 km from the first samples) were submitted in May 2009. One sample had been fixed in 96% ethanol, the other fixed in 10% formalin. Due to the poor quality of the samples, the fluorescent antibody test was inconclusive. RNA was extracted from both samples using the TRIzol[®] method and subjected to real-time reverse transcription polymerase

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chain reaction (RT-PCR) for rabies [16]. This assay detected the rabies virus genome in the ethanol-fixed sample but not the formalin-fixed sample (Fig. 1). Nested RT-PCR also generated an amplicon (Fig. 1, inset) that was sequenced to confirm amplification of the rabies virus genome from this sample. Detection of rabies virus in decomposed samples by RT-PCR has already been demonstrated from an Ethiopian wolf carcass [17] in which the brain was stored in 10% dimethylsulphoxide. This is the first observation to our knowledge of successful molecular detection of rabies virus RNA from a diagnostic sample fixed in ethanol.

Phylogenetic comparison of all five sequences from the 2008/9 outbreak with others derived from Ethiopia (Table 1) and including the sequence from the 2003/4 rabies outbreak [10] demonstrated that the sequences derived from the 2008/9 samples are identical for the 400-base-pair fragment aligned (Fig. 2). All wolf samples were obtained from within the Bale Mountains National Park (Fig. 2, inset). The wolf-derived sequences clustered with other rabies viruses reported from other locations within Ethiopia. The comparison of a 400-bp region indicated that the 2009 rabies virus genome sequence was 1.2% divergent from sequences obtained from the previous outbreak in 2003/04 (AY500827) but was less divergent (0.4%) from rabies virus sequences derived from dogs in Ethiopia (EU853580/EU853581). It is noteworthy that these latter sequences were all obtained 20 years previously, between 1987 and 1988, and from areas outside the Bale Mountains [14]. The lack of information on the rabies isolates currently circulating within Ethiopia precludes conclusions

being drawn on why the 2008/9 outbreak virus appears to be phylogenetically closer to viruses detected 20 years ago than those detected in the Bale Mountains in 2003. The viruses present in Ethiopia belong to the cosmopolitan strain of rabies that has proven very efficient in spreading throughout the world over the last 200 years [14]. There is also evidence that the rabies viruses within Ethiopia are closely related to those in neighboring Sudan and that some exchange occurs [3].

This study reports evidence of a further epizootic of rabies within the wolf packs of the Bale Mountains. Phylogenetic analysis demonstrates that a virus detected in the May 2009 sample is identical to those found in October 2008 samples. The likely source of the October cases is the dog reservoir in and around the Bale Mountains, although it is unclear whether the May 2009 case occurred due to wolf-to-wolf transmission from the earlier outbreak in the Web Valley despite a vaccination intervention or was a repeated spillover from the dog population. Further phylogenetic studies involving well-characterised samples from all potential rabies vectors in Ethiopia may define the source of the disease in Ethiopian wolves more accurately. This knowledge would directly inform attempts to protect this threatened carnivore population. Such investigations have not been conducted in this region of Africa but would be beneficial in attempting to control rabies within both wild and domestic animal populations.

Parenteral vaccination interventions have offered the possibility of protecting an unexposed wolf pack via a cordon sanitaire or quarantine line [2, 11], although in order to provide continued protection, inoculations will

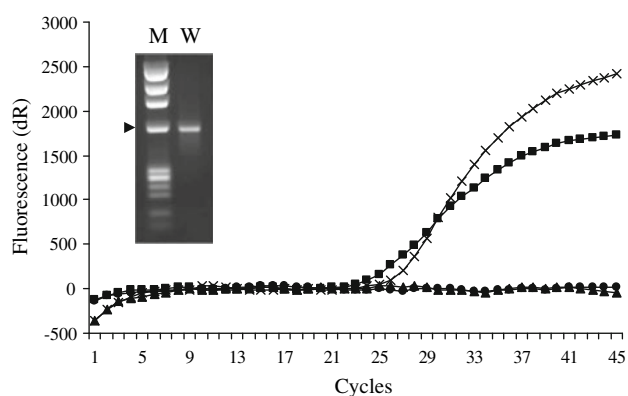


Fig. 1 Detection of rabies virus in an ethanol-fixed brain sample by real-time RT-PCR. Two RNA samples were tested: one had been fixed in ethanol (*crosses*), the other fixed in formaldehyde (*triangles*). Also shown are the rabies genotype 1 positive control (*squares*) and no-template control (*circles*). The inset shows the amplicon generated by nested RT-PCR from the ethanol-treated brain sample (*W*). The band was sized by comparison with DNA markers (*M*); the arrow indicates the 603-base-pair marker

Table 1 Details of rabies virus sequences used in this study

GenBank accession number	Host species	Year	Reference
EW_SAF_1_08 ^a	Ethiopian wolf	2008	This study
EW_JF_2_08 ^a	Ethiopian wolf	2008	This study
EW_AM_3_08 ^a	Ethiopian wolf	2008	This study
EW_SM_4_08 ^a	Ethiopian wolf	2008	This study
EW_1_09 ^a (GU062189)	Ethiopian wolf	2009	This study
EU853581	Dog	1988	[14]
EU853580	Cow	1987	[14]
AY502132	Jackal	?	[3]
AY103015	Jackal	?	[3]
U22637	Dog	1988	[4]
AY500827	Ethiopian wolf	2003	[10]
AY502128	Dog	2001	[3]
AY502125	Dog	2001	[3]

The challenge virus standard (CVS) was used as an outgroup

^a These sequences were identical over 400 base pairs of the nucleoprotein coding gene

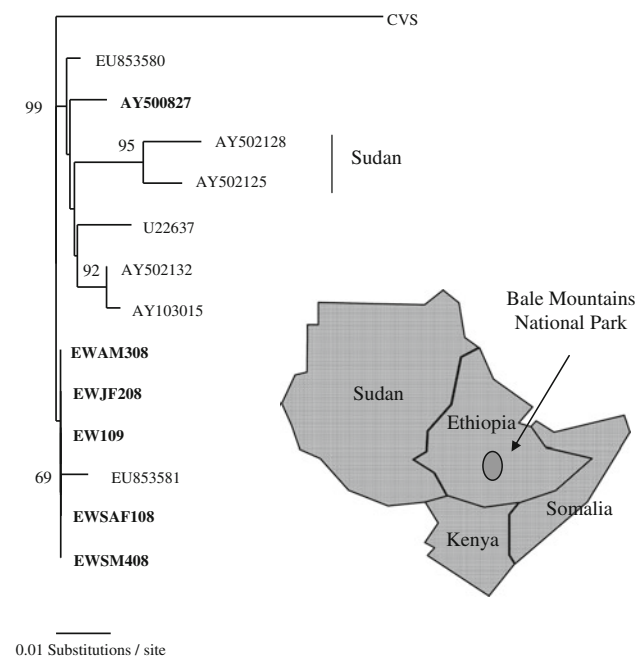


Fig. 2 Neighbour-joining phylogenetic analysis of rabies viruses from northeast Africa, including the current sequence (GU062189) derived from five Ethiopian wolf carcasses, the sequence from a previous outbreak (AY500827) and previously reported isolates from Ethiopia and Sudan. Sequences from Ethiopian wolf samples are shown in *bold*. Bootstrap values greater than 65, calculated from 100 replicates using the maximum-likelihood algorithm, are shown, and the challenge virus standard (CVS) has been used as an outgroup. A map of Ethiopia (*inset*) showing the location of the Bale Mountains National Park is included in the figure

need to be repeated to ensure that the basic reproductive number (R_0) of the virus is <1 , with the aim that rabies will be eliminated. In response to the 2008/9 outbreaks, 98 wolves from 20 packs were vaccinated, and domestic dog vaccination continues in and around Bale. However, in order to be effective, dog vaccination would need to be extended, and other potential reservoirs of rabies virus, particularly golden jackals (*Canis aureus*), should also be considered in future vaccination strategies if the Ethiopian wolf is to be protected from further rabies outbreaks. Vaccination through oral baiting may provide an alternative approach to establishing quarantine lines.

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