Rabies virus strains circulating in Bhutan: implications for control

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Epidemiology and Infection **(2011); 139: 1457–1462**

Available online at: http://dx.doi.org/10.1017/S0950268810002682 (paywalled)

Summary

We report a molecular epidemiological study of rabies virus strains circulating in animal populations in Bhutan, and investigate potential origins of these viruses. Twenty-three rabies virus isolates originating from dogs and other domestic animals were characterized by sequencing the partial nucleoprotein (N) gene (395 *bp)*. Phylogenetic analysis was conducted and the Bhutanese isolates were compared with rabies viruses originating from other parts of the world. Phylogenetic analysis showed that Bhutanese isolates were highly similar and were closely related to Indian strains and South Asian Arctic–like–1 viruses. Our study suggests that the rabies viruses spreading in southern parts of Bhutan have originated from a common ancestor, perhaps from the Indian virus strain.

Keywords: molecular epidemiology, rabies, Arctic–like virus, Bhutan

Rabies is caused by a virus within the genus *Lyssavirus* of the family *Rhabdoviridae.* According to the official *ICTV* Master Species List 2009-version 6, the genus *Lyssavirus* is composed of twelve species: Aravan virus (ARAV), Australian bat lyssavirus (ABLV), Duvenhage virus (DUUV), European bat lyssavirus 1 (EBLV-1), European bat lyssavirus 2 (EBLV-2), Irkut virus (IRKV), Khujand virus (KHUV),

Lagos bat virus (LBV), Mokola virus (MOKV), rabies virus (RABV), West Caucasian bat virus (WCBV), and Shimoni bat virus (SHIBV) (ICTV 2009; ICTV 2011). RABV is the only lyssavirus present in terrestrial mammals throughout the world and associated with bats only in the Americas.

The RABV genome consists of a single-stranded, non–segmented, negative-sense RNA of approximately 12 kb in size and encodes for five structural proteins: the nucleoprotein (N protein), phosphoprotein (P protein), matrix protein (M protein), glycoprotein (G protein), and the RNA-dependent RNA-polymerase (L protein). The N, P, and L proteins form the internal helically packaged ribonucleocapsid complex (RNP) whilst the M and G proteins form the inner and outer lipid-bilayer envelopes surrounding the RNP core, respectively (Wunner et al., 1988). Many of these genes have been targeted for molecular studies. The N genes are highly conserved and have been extensively employed for rabies diagnosis using RT-PCR and other genetic analyses (Smith et al., 1992; Kuzmin et al., 2004; Denduangboripant et al., 2005; Bourhy et al., 2008). These studies have provided clearer understanding about the epidemiology of rabies virus distribution in the world.

Rabies is a fatal zoonotic disease. It is endemic in Asia, where it causes about 31,500 human deaths each year despite the availability of effective vaccines (Knobel et al., 2005). Of these cases, an estimated 20,000 human deaths occur every year in India alone. Lack of comprehensive rabies control programs and an inability to pay for post exposure treatments after dog bites are responsible for high incidences of rabies in Asian countries (Knobel et al., 2005; Wilde et al., 2007). Over the past decade many molecular epidemiological studies have been conducted in Asian countries, and have provided a better understanding of the RABV variants circulating in Asia and the transmission dynamics of the disease (Denduangboripant et al., 2005; Nanayakkara et al., 2003; Nagarajan et al., 2006; Susetya et al., 2008; Gong et al., 2010; Hyun et al., 2005). This information is needed for implementation of effective rabies control programs in the region.

Bhutan is located between China and India and canine rabies is prevalent in southern Bhutan along the border with India (Tenzin et al., 2010). As in other Asian countries, domestic dogs in Bhutan play an important role in the maintenance and transmission of the disease to other domestic animals and occasionally to humans. In recent years, rabies outbreaks have increasingly occurred in endemic southern Bhutan as well as in some other areas where rabies had not been reported previously (Tenzin et al., 2010). Despite frequent outbreaks, molecular epidemiological studies of rabies in Bhutan have not been undertaken and the rabies virus variants circulating in the country are unknown.

In the present study, we performed genetic characterization of RABV based on the partial nucleoprotein (N) gene. A phylogenetic analysis of these N gene sequences was performed to investigate their genetic relationship with other RABV variants circulating in the world, especially in Asia. The information generated from this research could help in planning a more effective rabies control program in Bhutan.

Twenty-three fresh brain tissue samples were obtained from cattle (16), dogs (4), cat (1), and pigs (2) that died of clinically confirmed rabies from four southern districts (Samtse, Chhukha, Sarpang,

and Samdrup Jongkhar) in Bhutan during 2008 and 2009 (Table 8.1, Figure 8.1). These samples were collected from areas near the border between Bhutan and the Indian states of Assam and West Bengal. Rabies was confirmed in each case by the fluorescent-antibody test (FAT). For RT-PCR and nucleotide sequencing, about 10 mg of each FAT-positive brain tissue sample was smeared onto FTA® Gene Guard System (a commercial product consisting of filter paper impregnated with patented chemicals supplied by Whatman, USA), air-dried, and then transferred to the laboratory (WHO Collaborating Centre for Research and Training on Viral Zoonoses, Faculty of Medicine, Chulalongkorn University) in Thailand.

The brain tissue sample from the filter paper was eluted by rocking each dried brain spot in 9 ml of lysis buffer (NucliSens; BioMerieux) at room temperature with a rotator-mixer (rotor size, 60; BioSan) at 60 rpm for a period of 2 hours. The filter paper was then removed from the buffer solution.

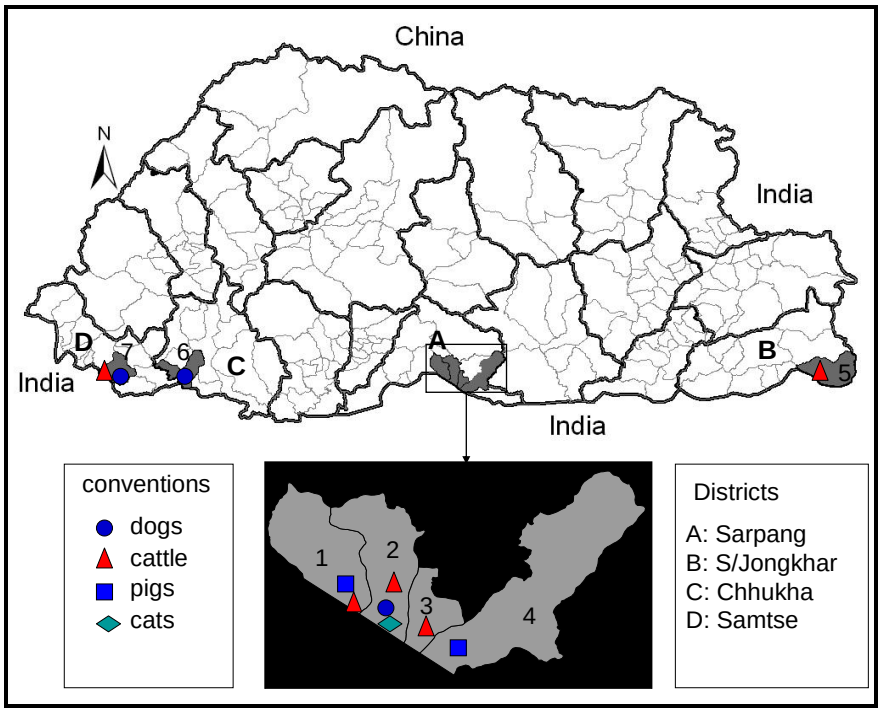

Figure 1: Map of Bhutan showing the geographical locations where the RABV isolates were obtained from different species of animals. Thick and thin lines on the map indicate the district and sub-district boundaries, respectively. Numerical values on the map indicate the names of sub-districts where RABV isolates were obtained (1) Bhur; (2) Gelephu; (3) Chhuzargang; (4) Umling; (5) Langchenphu; (6) Phuentsholing; (7) Samtse; see Table 1 for details). Symbols represent the species from which the virus was isolated.

The total RNA extraction from eluted specimens was performed by using a silica-guanidine thiocyanate protocol, NucliSense isolation reagent (Biomerieux, Boxtel, The Netherlands) according to the manufacturer instructions.

Single-step reverse-transcription polymerase chain reaction (RT-PCR) was performed using the One Step RT-PCR Kit (Qiagen GmbH). Specific sense primer CN8 (3' GT(TC) GGA TGT TAT ATG GG 5', nt 1013 - nt 1029) and an antisense primer CN4 (3' GGA TTG AC(AG) AAG ATC TTG CTC AT 5', nt 1514-nt 1536) were used for the N gene amplification. Binding sites of the primers were referred to the positions of total genomic sequence of the Pasteur Virus (PV) strain of RABV (GenBank accession no. M13215). One cycle of reverse transcription was done at 50°C for 35 min, followed by denaturation at 95°C for 15 min. PCR was followed by 40 amplification cycles of denaturation at 94°C for 1 min, primer annealing at 50°C for 1 min, and extension at 72°C for 1 min. Finally a 10 min extension step at 72°C was done to complete the amplification of the target gene. The final PCR products were run in a 2% agarose gel electrophoresis in TBE 1x buffer stained with ethidium bromide (at 1µg/ µl), and viewed under UV light to observe the specific bands (Hemachudha et al., 2003).

Gel slices containing RT-PCR products were excised from the gel and the RT-PCR products were purified using QIAquick PCR Purification Kit (Qiagen Ltd., Crawley, UK) according to the manufacturer's instruction. Direct sequencing of the N gene was performed using primers CN4 and CN8 with ABI PRISM Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and ABI PRISM 310 DNA sequencer (Applied Biosystems, Foster City, CA, USA) (Hemachudha et al., 2003). For each RABV isolate, 395 nucleotides of N gene corresponding to position 1101 – 1495 of the Pasteur Virus genome (GenBank accession no. M13215) were analysed. All nucleotide sequences generated in this study were submitted to GenBank and their accession numbers were assigned (Table 1).

Multiple sequence alignment was performed by using MUSCLE version 3.8 program (Edgar 2004). A neighbour-joining (NJ) analysis employing Kimura-2 parameter model with bootstrap statistic test of the phylogenetic tree (1000-replicates) was performed in MEGA version 4.0.2 program (Tamura et al., 2007). Thirty four additional N gene sequences of RABV previously published were retrieved from GenBank and used for comparison. The GenBank accession numbers and other details of the sequences are shown in Figure 8.2. The bootstrap values of >50% are shown on the tree branches. Tree Explorer module in MEGA 4.0 was used to obtain the graphic output. Phylogenetic analysis using Bayesian Markov Chain Monte Carlo (MCMC) method was also implemented in Mr Bayes version 3.1 program (Huelsenbeck et al., 2001) with a *GTR+I+gamma* evolutionary model. The analysis was run for 2,000,000 generations to get 200,000 samples from the posterior probability distribution. The posterior probability values >0.95 was considered significant. The estimated tree topology was illustrated using Tree view program.

The geographical distribution of the Bhutanese rabies isolates were mapped using a Bhutan boundary shape files in ArcGIS 9.3 (ESRI, Redlands, USA, CA).

Table 1: Rabies virus isolates obtained from Bhutan and used in the present study

Isolate ID	District	Sub-district	Location	Host species	Year of isolation	GenBank accession
BHT 2445	Sarpang	Gelephu	Puranobasti	Cattle	2008	HQ166002
BHT 2685	Sarpang	Gelephu	Tankabasti	Cattle	2008	HQ166003
BHT 88	Sarpang	Gelephu	Lodrai	Dog	2008	HQ166004
BHT 183	Sarpang	Gelephu	Lekithang	Cattle	2008	HQ166005
BHT 187	Sarpang	Bhur	Majuwa	Cattle	2008	HQ166006
BHT 202	Sarpang	Bhur	Majuwa	Pig	2008	HQ166007
BHT 220	Sarpang	Gelephu	Majuwa	Cattle	2008	HQ166008
BHT 317	Sarpang	Gelephu	Lekithang	Cattle	2008	HQ166009
BHT 573	Sarpang	Gelephu	Pelrithang	Cattle	2008	HQ166010
BHT 333	Sarpang	Bhur	Jarwa	Cattle	2008	HQ166011
BHT 2	Sarpang	Gelephu	unknown	unknown	2008	HQ166012
BHT 101	Sarpang	Gelephu	unknown	unknown	2008	HQ166013
BHT 2000	Sarpang	Gelephu	Tankabasti	Dog	2009	HQ166014
BHT 2053	Sarpang	Gelephu	Puranobasti	Cattle	2009	HQ166015
BHT 2169	Sarpang	Chhuzargang	Pemaling	Cattle	2009	HQ166016
BHT 2196	Sarpang	Chhuzargang	Chhuzargang	Cattle	2009	HQ166017
BHT 2719	Sarpang	Umling	Dungmin	Pig	2009	HQ166018
BHT 630	Sarpang	Gelephu	Puranobasti	Cat	2008	HQ166019
BHT 631	Samtse	Samtse	Gerigoan	Cattle	2008	HQ166020
BHT 68	Samtse	Samtse	Lamatar	Dog	2009	HQ166021
BHT 481	Samdrup Jongkhar	Langchenphu	Talabasti	Cattle	2009	HQ166022
BHT 469	Samdrup Jongkhar	Langchenphu	Talabasti	Cattle	2009	HQ166023
BHT 450	Chhukha	Phuentsholing	P/ling town	Dog	2009	HQ166024

Figure 2 shows the Bayesian phylogenetic tree inferred by comparing the partial N gene sequences of 23 Bhutanese isolates with 34 RABV N gene sequences available in GenBank.

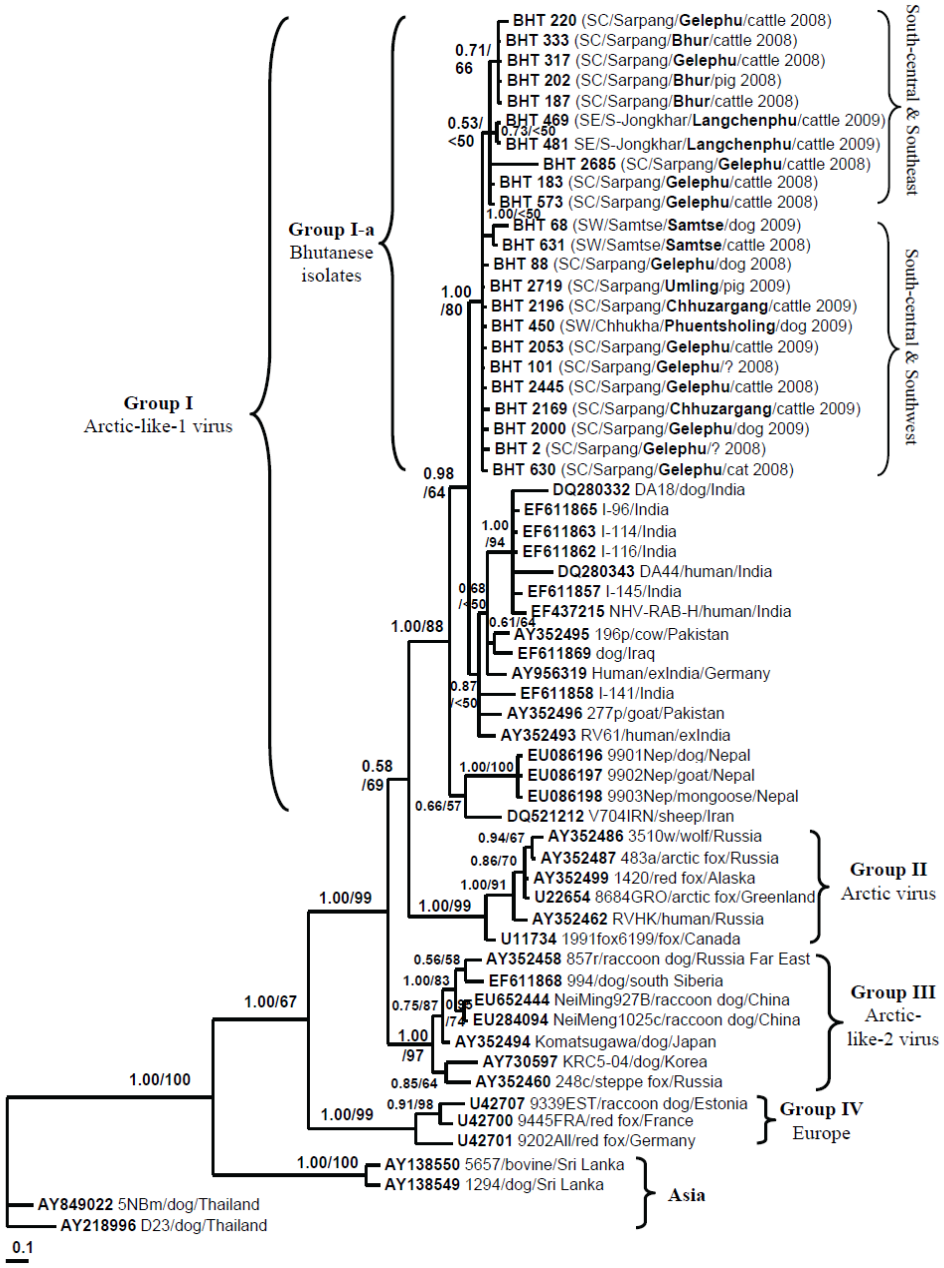

Figure 2: A phylogenetic tree of 60 RABV isolates analysed in this study. The tree was inferred by the Bayesian MCMC phylogenetic analysis method using a partial nucleoprotein (N) gene (395 *bp*) of 23 Bhutanese isolates compared to other N rabies sequences obtained from the GenBank database. The posterior probability value of each node is shown along the tree branches. Bootstrap branch-support values >50% (with 1000 replicates) generated from neighbour-joining (NJ) analysis is also indicated. A scale of base substitution number per site is shown at the bottom of the tree. Additional information of each Bhutanese sequence is mapped in parentheses as region/district/sub-district/host species/year of isolation (SW, Southwest; SC, South-central; SE, Southeast). The isolate ID, host species, country of isolation, and GenBank accession numbers of other rabies sequences are also given for examination. Various groupings found in this study are described in the text.

The topology of the NJ tree (result not shown) was similar to that of the Bayesian phylogeny and only its bootstrap values were mapped on the Bayesian tree. The tree shows four significant genogroups which was supported with posterior probability values >0.95. Group I consisted of RABV isolates from Iraq, Iran, Pakistan, India, and Nepal with 88% bootstrap supporting value. These isolates were previously described as Arctic–like–1 viruses circulating in the Middle East and South Asia (Kuzmin et al., 2004; Nadin-Davis et al., 2007; Kuzmin et al., 2008; Mansfield et al., 2006). The Bhutanese isolates formed a monophyletic cluster (labelled as Group I–a) within this Arctic–like–1 virus group. Group II is composed of six Arctic virus isolates originating from Russia, Canada, Alaska, and Greenland (bootstrap value of 99%). Group III consisted of seven viruses originating from Korea, Japan (formerly), southern Siberia, Far East Russia, and northern China (Inner Mongolia) with 97% bootstrap value. These isolates were previously described as an Arctic–like–2 viruses circulating in Northeastern Asia (Hyun et al., 2005; Kuzmin et al., 2004; Nadin-Davis et al., 2007; Kuzmin et al., 2008). Three isolates from Europe formed Group IV with 99% bootstrap support value, and the other four isolates from Asia (Thailand and Sri Lanka) formed separate clusters according to the country of origin. The phylogenetic analysis clearly revealed that all Bhutanese isolates were closely related to Indian strains and formed a large cluster with the Arctic–like–1 viruses, and were distinct from other Asian rabies virus isolates from Thailand and Sri Lanka.

This is the first report characterizing the molecular epidemiology of rabies virus isolates in Bhutan. The result confirmed that all isolates from Bhutan belong to the rabies virus and were not related to other rabies related viruses (e.g. from bats) of *Lyssavirus* genus. Our analysis showed that the Bhutanese isolates were highly similar and did not form any distinct subgroups although the isolates originated from different geographical areas. This suggests that the rabies virus variants circulating in southern Bhutan originated from a single common ancestor. Even though the Bhutanese isolates did not form clear subgroups due to low bootstrap values along most branches, they tended to separate into two subgroups based on the geographical locations of the isolates: the Southeast-and-Central and Southwest-and-Central subgroups (Figure 2, see Table 1 for details). However, more sequences from both Southeast and Southwest areas of Bhutan would be needed to provide greater phylogenetic support and confirm our hypothesis.

Phylogenetic analyses revealed that Bhutanese RABV isolates were more closely related to the RABV strains from India and Arctic–like–1 viruses circulating in South Asia (Nadin-Davis et al., 2007; Kuzmin et al., 2008), and could be grouped together as a large cluster of the South Asian Arctic–like–1 viruses (Group I in Figure 2). Geographically, Bhutan lies in the same Himalayan region of the Indian sub-continent where the emergence and extensive circulation of the Arctic–like–1 rabies viruses has been confirmed in India (Nadin-Davis et al., 2007). However, it should be noted that the Indian rabies sequences used for comparison in this study originated mostly from the southern part of India and ex-India (a foreigner who died of confirmed rabies after being bitten by a dog in southern India). Even though South India is quite far away from Bhutan, it is interesting to observe that Bhutanese and Indian RABV isolates share close genetic relationships, and suggests that they have originated from a common ancestor, probably the Indian strain. Translocation of infected dogs via human activities may be responsible for the spread of rabies virus between the two countries. In studies (Denduanboripant et al., 2005; Susetya et al., 2008; Gong et al., 2010), the translocation of dogs together with their owners migrating for work and trades purposes have been hypothesized as a probable mechanisms for the spread of rabies virus from country-to-country or region-to-region in some Southeast Asian countries (e.g. in Thailand, Vietnam and Indonesia).

In this study, a direct comparison between rabies isolates of Bhutan and those from Northeast India (which share a porous border) was not possible because there are no published sequence data available from Northeast India. Perhaps a similar rabies virus variant is circulating in dogs in Northeast India. This hypothesis needs to be confirmed by surveillance and conducting molecular studies and genetic analyses.

The limited distribution of rabies virus within the southern areas of Bhutan provides important information for planning and implementing a rabies control program in the country. It implies that a successful control and even elimination of rabies is promisingly practical in Bhutan if a sustained vaccination campaign of domestic dogs (>70% coverage) and dog population management is carried out efficiently. Control of rabies within the domestic dog cycle would break the chain of transmission (spill over) into other animals, and would eventually eliminate the disease from the country.

In conclusion, we recommend that more sampling of virus from potential reservoirs from different rabies outbreak areas and time periods is needed to provide detailed information about rabies virus transmission dynamics in Bhutan.

Acknowledgements

We thank The Thailand Research Fund (grant No.DBG5180026) for partial funding of this research work. We would also like to thank Jambay Dorjee, Kesang Jigme and Lungten for their gift of the brain tissue specimen from south east and south west Bhutan.

Declaration of interest: None

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