Arch Virol (2011) 156:681–684
DOI 10.1007/s00705-010-0868-9

BRIEF REPORT

Characterization of rabies virus from a human case in Nepal

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Received: 20 August 2010 / Accepted: 18 November 2010 / Published online: 6 February 2011

Abstract Rabies is endemic throughout most of Asia, with the majority of human cases transmitted by domestic dogs (*Canis familiaris*). Here, we report a case of rabies in a 12-year-old girl in the Lalitpur district of Nepal that might have been prevented by better public awareness and timely post-exposure prophylaxis. Molecular characterization of the virus showed 100% identity over a partial nucleoprotein gene sequence to previous isolates from Nepal belonging to the 'arctic-like' lineage of rabies virus. Sequence analysis of both partial nucleoprotein and glycoprotein genes showed differences in consensus sequence after passage *in vitro* but not after passage *in vivo*.

Rabies is a neglected zoonosis, with the majority of the estimated 24,000-93,000 human deaths due to rabies per year occurring in rural parts of developing countries [1]. This large number of deaths is particularly disturbing, considering that rabies is a preventable disease. Although many cases can be attributed to poor access to medical resources, failure to present for timely post exposure prophylaxis (PEP) is still the root cause of many deaths [2], as illustrated by the following case in a young girl from the Lalitpur district of Nepal.

A 12-year-old girl was admitted to Patan Hospital on July 10, 2003, with fever, malaise and nausea. She had a three-day history of right-arm pain, restlessness, vomiting and headache. Her condition deteriorated rapidly after admission, and she became distressed and hyperactive. She developed aerophobia, hydrophobia and hypersalivation and died on the second day after admission.

Three months previously, she had been bitten by a pet dog that had subsequently been killed due to its aggressive nature. She had also been in contact with an ownerless dog in the same period but had told neither her parents nor teachers about either incident and was therefore not given PEP. The wounds had remained untreated, but she had consulted a traditional faith healer the day before admission to hospital.

Brain samples were taken post-mortem and submitted to the Central Veterinary Laboratory, Kathmandu. Although the Seller's stain test for detection of Negri bodies undertaken on original brain material was negative, a fluorescent antibody test (FAT) and a mouse inoculation test (MIT) were positive. Seller's stain on the brains harvested from the mice used in the MIT were positive for rabies [3]. All family members were given rabies vaccination as a precaution after diagnosis was confirmed. This report describes the further investigation and molecular characterization of the virus.

Virus was passaged in mice for potential vaccine production at the Rabies Vaccine Production Laboratory, Kathmandu, in 2003. Thirty microlitres of a 10% brain homogenate with PBS, containing penicillin and streptomycin (*GIBCO*), were inoculated intracerebrally into

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twelve three- to four-week-old mice [4]. Inoculated mice were observed daily and humanely killed at the onset of paralysis. A 20% suspension of infected mouse brain in 2% horse serum was inoculated into a second group of mice and this process was repeated for a further 10 passages with 10-15 mice per passage. Infected human brain homogenate was also inoculated into BSR cells and passaged four times. Original human brain suspension, tenth-passage mouse brain suspension and fourth-passage BSR cell tissue culture supernatant (TCSN) were all tested using a rabies antigen detection test kit (Rapid Antigen Detection Kit, Animal Genetics Inc.) according to the recommended protocol.

Viral RNA was then extracted in duplicate from human brain and a tenth-passage mouse brain homogenate using TRIzol^{TM} (Invitrogen), and from fourth passage BSR cell TCSN using TRIzol LS^{TM}. RNA was reverse transcribed, and a 606-base-pair region of the gene encoding the nucleoprotein (N-gene), and a two-kilobase-pair region spanning the gene encoding the glycoprotein (G-gene) were amplified by polymerase chain reaction (RT-PCR) using techniques described previously [5]. PCR products were purified using a gel extraction kit (QIAGEN) and then sequenced using a BigDye^{TM} terminator sequencing kit and an ABI Prism 3130 analyzer (Applied Biosystems) using primers described previously [6]. Sequence assembly and consensus determination for a 400-base-pair region of the N-gene (GenBank accession number HQ913571) and a 1518-base-pair region of the G-gene (GenBank accession number HQ913570) were undertaken using Seqman (Lasergene 7, DNAstar). Sequences were aligned using CLUSTALX2.

The mean onset of paralysis decreased in mice from 9 days (SD 1 day) in the second passage to 6 days (SD 0 days) by the tenth passage. Clinical signs in otherwise healthy mice were considered indicative of rabies encephalitis, and human brain, tenth-passage mouse brain and fourth-passage BSR TCSN were all positive using the Rapid Antigen Detection Kit and by PCR.

At least two primer sequences, one forward and one reverse, were used to generate each consensus sequence, and in all cases, the consensus was unambiguous. The sequences derived from human brain and tenth-passage mouse brain were identical, but the sequence from the fourth-passage tissue culture supernatant showed a single synonymous nucleotide transition at nucleotide position 69 (G-A) in the N-gene and three non-synonymous mutations in the G-gene, corresponding to substitutions at amino acid positions 301(L-H), 303 (H-R) and 425 (K-E) of the glycoprotein ectodomain.

The virus isolated from this case is 100% identical, over the N-gene region studied, to viruses isolated from two dogs and a mongoose (EU086198) in Nepal. Phylogenetic analysis showed these viruses to be in one distinct group within arctic-like lineage 1 [7, 8]. There is weaker support for an apparent monophyletic grouping of arctic-like virus lineage 1 with the 'true' arctic lineage, distinct from arctic-like lineage 2. These arctic and arctic-like viruses are more similar to the cosmopolitan lineage circulating in Europe than Asian-lineage viruses isolated in China, Thailand, Malaysia and the Philippines (Fig. 1)

Here, we have characterized a virus from a child who died from rabies without receiving PEP. Phylogenetic analysis showed that this virus was most similar to rabies viruses isolated from dogs in Nepal. As in all countries where rabies is endemic in the dog population, the majority of human rabies cases, as well as the 30,000 doses of PEP administered each year in Nepal, are subsequent to dog bites [9]. This close association with dog isolates concurs with the case history, suggesting the most likely source of infection for this girl was the pet dog that was killed for aggressive behaviour, a key sign of rabies in animals. Had that dog been tested, or if rabies had even been suspected, it is possible the patient would have received PEP.

All of the isolates in this study from Nepal, including this human case, are part of a lineage of viruses that are thought to have evolved approximately five hundred years ago (95% HPD 242-750) from viruses circulating among arctic foxes in the polar and sub-polar regions [8]. These arctic-like viruses are now widely distributed in the Middle East, and southern and eastern Asia [7, 8, 10]. Our analyses show strong support for a separate origin of arctic-like viruses to viruses circulating through the rest of South East Asia. In addition, our analyses suggest that there are two distinct clades within the arctic-like group 1. This concurs with analyses from previous studies [7, 8] and suggests that viruses isolated from Nepal are distinct from arctic-like viruses from India and Pakistan. Further analysis of a larger number of samples from Nepal and neighbouring regions of India would reduce sampling bias and could confirm this. Control and even elimination of canine rabies in Nepal would be much more feasible if there were not regular movement of disease across land borders.

Rabies in Nepal is thought to circulate in two epidemiological cycles: an urban cycle involving maintenance of infection in dog populations and a sylvatic cycle involving mongooses (family *Herpestidae*), jackals (*Canis aureus*), and wolves (*Canis lupus*) [9]. The single mongoose isolate in our data set clusters with the Nepalese dog isolates, but this could be an isolated incidence of a mongoose interacting with an infected dog. There is no surveillance system for sylvatic rabies in Nepal, and therefore the true incidence in those species is unknown, and the availability of samples is limited. Further sampling and molecular characterization of both domestic animal and wildlife rabies isolates would resolve this issue.

The virus isolated from this patient was passaged in mice and tissue culture for potential vaccine production. Cell-culture-derived vaccines are recommended over

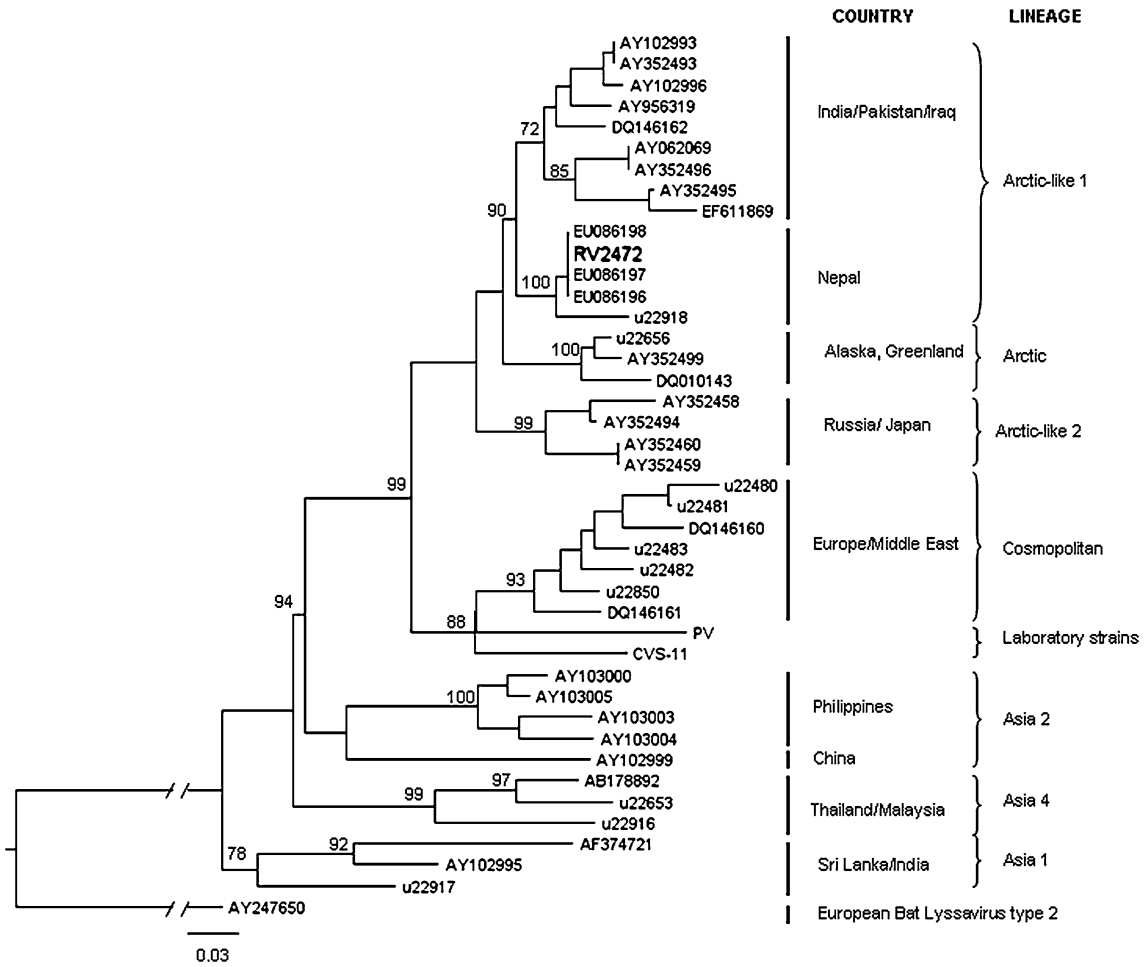

Fig. 1 Phylogenetic tree of 41 Asian rabies virus isolates, using the neighbour-joining algorithm to analyze a 400-bp region of the nucleoprotein gene, bootstrapped 100 times and visualized using Figtree. Boostrap values greater than 70 at key nodes are shown. The virus isolated from this case is highlighted in bold. The tree is rooted with European bat lyssavirus included as an outgroup. Lineages are labelled according to Ref. [8]

neural-tissue-derived vaccines for their increased safety and efficacy [11]. Adaptation to cell culture can select for mutations that can change the phenotype of a virus [12], which is relevant to its use in a vaccine. The sequence of the glycoprotein gene after cell culture showed three non-synonymous changes. None of those amino acid mutations were in regions considered antigenically important based on monoclonal antibody escape mutants, and they were different from those reported in other studies [12]. Quantification of any antigenic differences to wild-type strains would help determine its suitability as a vaccine candidate [13].

The virus isolate remained virulent after repeated passage in mice, with no change in consensus sequence in the regions of the N-gene and G-gene studied. In contrast, a previous study of rabies virus genetic variation demonstrated a single nucleotide substitution in the G-gene consensus sequence, corresponding to an amino acid substitution (aspartate to glutamic acid at position 247 in the glycoprotein), after three passages in adult mouse brain, and no nucleotide changes in the N-gene consensus sequence after 20 passages in BSR cells [14]. Possible reasons for the contrast include differences in genetic polymorphism in the original viral isolates and differences in cell culture technique.

Rabies is a major public health problem throughout Asia. Although this case occurred several years ago, without a coherent control strategy, rabies continues to be a concern in Nepal. The published figure of 100 human deaths per year in Nepal is likely to be a gross underestimate [15]. Comparison of reported cases with active surveillance [16] and models of rabies incidence based on dog bites [1] suggest that the true incidence of rabies may be 100 times what is reported to authorities. Rabies in animals in Nepal may also be underreported, with southern districts reporting more cases than northern ones, and large ruminants overrepresented, at 56% of reported cases [3].

Control of rabies first requires accurate knowledge of its incidence and epidemiology, to focus vaccination

campaigns and control methods. Where veterinary and laboratory services are scarce, collaboration is the key to increasing that knowledge. The production of a cost-effective, local vaccine that meets global safety and efficacy standards is then of major importance for implementing and maintaining those vaccination campaigns. This report also illustrates the importance of public education regarding rabies and the advantages of collaboration between veterinary and public health services in a 'one health' approach.

Acknowledgements The authors would like to thank the Central Veterinary Laboratory, Kathmandu, for providing samples, and Mrs. Mangala Manandhar, Mr. Shri Ram Ghimire and Mr. Tul Bahadur Rai for technical assistance. Work undertaken at the Veterinary Laboratories Agency was funded by DEFRA project SEO423.

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