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Research paper

Evidence of two distinct phylogenetic lineages of dog rabies virus circulating in Cambodia

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ABSTRACT

This first extensive retrospective study of the molecular epidemiology of dog rabies in Cambodia included 149 rabies virus (RABV) entire nucleoprotein sequences obtained from 1998–2011. The sequences were analyzed in conjunction with RABVs from other Asian countries. Phylogenetic reconstruction confirmed the South-East Asian phylogenetic clade comprising viruses from Cambodia, Vietnam, Thailand, Laos and Myanmar. The present study represents the first attempt to classify the phylogenetic lineages inside this clade, resulting in the confirmation that all the Cambodian viruses belonged to the South-East Asian (SEA) clade. Three distinct phylogenetic lineages in the region were established with the majority of viruses from Cambodia closely related to viruses from Thailand, Laos and Vietnam, forming the geographically widespread phylogenetic lineage SEA1. A South-East Asian lineage SEA2 comprised two viruses from Cambodia was identified, which shared a common ancestor with RABVs originating from Laos. Viruses from Myanmar formed separate phylogenetic lineages within the major SEA clade. Bayesian molecular clock analysis suggested that the time to most recent common ancestor (TMRCA) of all Cambodian RABVs dated to around 1950. The TMRCA of the Cambodian SEA1 lineage was around 1964 and that of the SEA2 lineage was around 1953. The results identified three phylogenetically distinct and geographically separated lineages inside the earlier identified major SEA clade, covering at least five countries in the region. A greater understanding of the molecular epidemiology of rabies in South-East Asia is an important step to monitor progress on the efforts to control canine rabies in the region.

1. Introduction

The developing world is severely affected by rabies, causing deaths in humans as well as in animals and resulting in significant economic losses. Indeed, it is estimated that globally canine rabies causes approximately 59,000 human deaths, over 3.7 million disability-adjusted life years (DALYs) and 8.6 billion USD in economic losses annually (Hampson et al., 2015).

Rabies virus (RABV) belongs to the family *Rhabdoviridae* of the order *Mononegavirales* which comprises at least 10 genera (http://www.ictvonline.org). The *Lyssavirus* genus includes classical RABVs, found in a wide range of different animal species throughout the world. All 13 other species of *Lyssavirus* except two have been isolated from bats, originating from different locations in Europe, Asia, Africa and Australia. RABV has a negative-sense RNA genome of about 12 kb encoding five proteins (Tordo et al., 1986; Tordo and Kouknetzoff, 1993). The nucleoprotein gene (N-gene) is widely used for molecular characterization and phylogenetic analysis of RABVs (Kissi et al., 1995). The large number of N-gene sequences published makes this an obvious region of the genome to study (Johnson et al., 2002; Chupin et al., 2013). The N-gene is also suitable for estimations of evolutionary rate and subsequent analysis of evolutionary history (Holmes et al., 2002; Bourhy et al., 2008).

Most South-East Asian countries are affected by rabies, but with the exception of a few countries, there is limited accurate epidemiological information available. According to the World Organization for Animal Health (OIE), there were 15 animal rabies outbreaks recorded in Vietnam in 2012 with 86 rabies cases found in dogs in five different administrative divisions of the country. For Laos it was only mentioned that rabies was present in 2012 with no indication of the number of outbreaks/cases. In Myanmar, the number of human rabies cases per year

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was estimated at about 1000 while in Thailand it was less than 25 (Gongal and Wright, 2011).

The only reliable data on human and animal rabies in Cambodia are available from the Institut Pasteur in Cambodia (IPC) where rabies diagnosis activity has been performed routinely since 1998, but laboratory-confirmed cases of rabies in dogs were recorded since the 1970s (Reynes et al., 1999). In the second half of the 1970s, dogs which are the most important vector for human rabies in Asia (Dodet et al., 2001) almost disappeared from Cambodia because of starvation. However, dog numbers rebounded in subsequent decades and rabies consequently became a serious public health concern. During the mid-1980s, the number of patients who have sought medical assistance after a dog bite has been at least 4000 per year, but the number of reported rabies deaths has been very low, partly because of improved post-exposure treatment delivery. Deaths occurring at home are usually not reported to health services. From 1982 to 1991 a total of 5437 animal bites were recorded and 51 human rabies deaths were reported (WHO/Rab. Res./93.44). Since 1994, rabies stopped being a notifiable disease in Cambodia. Thus, the only data available after 1994 is from the IPC, which established a rabies post-exposure treatment centre in 1995 (Reynes et al., 1999). From mid-1995 to 2007, 149,224 post-exposure prophylaxis (PEP) treatments were provided at the IPC Rabies Prevention Centre (Reynes et al., 1999; Ly et al., 2009; WHO/EMC/ZOO/96.8) and still 67 fatal human cases were reported countrywide following dog bites.

During 1998–2007, 610 animal samples (49% of all specimens tested) originating mostly from dogs with suspected rabies were found rabies-positive (Ly et al., 2009). A predictive model established by Ly et al. (2009) estimated that 810 human rabies cases occurred in 2007 alone. In 2008–2013, rabies was recorded mostly in dogs (n = 932, 99.3%). Rabies cases in cats (n = 3, 0.3%), bovines (n = 3, 0.3%) and swine (n = 1, 0.1%) were detected in very rare occasions (Institut Pasteur in Cambodia, unpublished data).

At present, the dog population in Cambodia is estimated to exceed 5 million animals and nearly one-third of the 250 Cambodian households surveyed in a preliminary study could recall at least one dog bite experience during 2004–2009 (Institut Pasteur in Cambodia, unpublished). Overall, the risk proportion (total number dog bites/total human population) in Cambodia was 5.6% (75/1339). This equates to an annual incidence of 1120 dog bites per 100 000 people (Lunney et al., 2012). Other mammals like mongoose, bats or other wild animals are sometimes suspected in Asia to contribute to maintaining the circulation of rabies. In Cambodia, Reynes et al. (2004) found serological evidence of *Lyssavirus* infection in bats. Several studies on RABV molecular epidemiology in Asia (Bourhy et al., 2008), namely in Vietnam (Nguyen et al., 2011; Yamagata et al., 2007), Thailand (Ito et al., 1999) and Laos (Ahmed et al., 2015) were conducted previously.

The objective of the present study was to perform a retrospective molecular and phylogenetic characterization of RABVs from dogs originating from different parts of Cambodia, to compare them to strains originating from other South-East Asian countries and to analyze the circulation of dog RABVs in this region.

2. Materials and methods

2.1. Samples and viruses

Dog brain samples received by the IPC Virology Unit from different regions of Cambodia between 1998 and 2011 were included in the study. In total, 149 rabies-positive samples obtained from dogs originating from 20 administrative regions of Cambodia were selected by location and year of sampling (Supplementary Table 1). Dog's heads were usually referred to the IPC lab from people who were consulting for PEP following the animal bite. The animal samples were tested by a standard direct fluorescent antibody test (FAT) (Dean et al., 1996) using an anti-rabies nucleocapsid conjugate (Anti-Rabies Nucleocapsid Conjugate; lyophilizied, adsorbed #357-2112, Bio-Rad, Marnes-la-Coquette, France) according to the manufacturer's instructions.

2.2. RNA purification, reverse transcriptase PCR and nucleotide sequencing

The diagnostic method for the initial detection of the RABV from clinical samples used a hemi-nested RT-PCR targeting a conserved region of the L-gene to confirm results of FAT as previously published (Dacheux et al., 2008). The target size of amplicons obtained after the second round of PCR was approximately 250 base pairs. Negative and positive control samples were included successively for each extraction, reverse transcription, and for all PCR steps.

To generate amplicons for sequence analysis, two overlapping RT-PCRs were conducted targeting the N-gene (Yang et al., 2011), which has been extensively used for phylogenetic analyses of RABVs. The two sequencing PCRs (Table 1) were conducted using the same reaction conditions and cycling protocols as previously described (Dacheux et al., 2008). The first PCR, using the primers RVN-71F (5′-ATGGATGCCGACAA GATTGTATTC-3′) and RVN-1118R (5′-GAATTCCTCTCCCAGATAGCC-3′) generated a 1048 bp amplicon; the second PCR, using the primers RVN-1091F (5′-CTAGGGGGCTATCTGGGAGA-3′) and RVN-1562R (5′-CGGCCAGACCGGCTCTAACAC-3′) produced a 472 bp amplicon. The PCR products were sequenced by Sanger method at a commercial facility (Macrogen, Seoul, South Korea) and resulted in a combined N-gene sequence of 1492 nucleotides.

2.2.1. Phylogenetic analysis

The raw sequences were edited with CLC Genomic Workbench Version 3.6.1. Multiple sequence alignment was built with Clustal Omega tool and edited with Jalview package Version 2.8. (Waterhouse et al., 2009) and GeneDoc Version 2.6.002 (Nicholas et al., 1997). Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013). Entire and partial (positions 56 to 454 according to ERA vaccine strain, GenBank #EF206707) N-gene sequences of RABVs from Cambodia, Vietnam, China, Thailand, Laos and Myanmar as well as a rabies vaccine strain PV (an outgroup) were included in the study (Supplementary Table 2). To choose the most suitable model for phylogeny, a Model Selection analysis was performed with MEGA6 package (Nei and Kumar, 2000; Tamura et al., 2013).

Phylogenetic analyses were conducted on RABV sequences including selected sequences sourced from the GenBank Database. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1346 positions in the final dataset for entire N-gene sequences, and 381 positions in the final dataset for partial N-gene sequences. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

The evolutionary history of the entire N-gene sequences was inferred using the Maximum Likelihood method. The evolutionary distances were computed using the Tamura 3-parameter model (Tamura, 1992). The percentage of trees in which the associated taxa clustered

Table 1
PCR and sequencing primers used in this study.

No	Name of PCR primers	Sequence 5′–3′	References
PCR primers			
1	PV 05 m	ATG ACA GAC AAY YTG AAC AA	Dacheux et al. (2008)
2	PV 09	TGA CCA TTC CAR CAR GTN G	
3	PV 08	GGT CTG ATC TRT CWG ARY AAT A	
4	β-Taq1	TCACCCACACTGTGCCCATCTACGA	
5	β-Taq2	CAGCGGAACCGCTCATTGCCAATGG	
Sequencing primers			
1.	RVN-71F	ATGGATGCCGACAAGATTGTATTC	Yang et al. (2011)
2.	RVN-1118R	GAATTCCTCTCCCAGATAGCC	
3.	RVN-1091F	CTAGGGGGCTATCTGGGAGA	
4.	RVN-1562R	CGGCCAGACCGGCTCTAACAC	